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The *Journal of Pharmaceutical Sciences* considers manuscripts for publication in the following categories:

Review Articles—Summaries covering the more recent advances in an area in which the author has been actively engaged will be published as review articles. Such papers are intended to be critical evaluations of published reports which have previously appeared in the scientific literature.

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JOURNAL OF

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The Editor comments

FACTS OR THEORIES?

We have become somewhat disturbed by a recent trend which appears to be developing in scientific reports—at least as some are being published in THIS JOURNAL. A basic concept of the scientific approach is that conclusions arrived at must be adequately substantiated by the data which have been obtained during the course of the study. This thought was concisely and pointedly stated by the late A. J. Carlson when he remonstrated: "Keep your mouth shut and your pen dry until you know the facts."

Reports today, however, at least in some fields of research activity, seem to be tending away from this philosophy. In many instances an extremely limited amount of experimental data is presented, from which very elaborate theories, mechanisms, and assorted postulations are derived. We all recognize that adequate opportunity for discussing and interpreting results is a fundamental necessity of good report writing and publication. However, the presentation of extensive hypothetical speculations, followed by unsupported and often questionable conclusions, does not follow from this concept. In keeping with the traditional scientific approach, authors must strive at all times to confine their interpretations only to those aspects which are adequately supported by experimental evidence. In those cases where some speculation appears warranted it should be clearly indicated that such statements are indeed only plausible postulations.

Certainly, if data presented without conclusions represents sterile report writing, then theorizing without adequate experimentation constitutes abuse of the privilege of publication.

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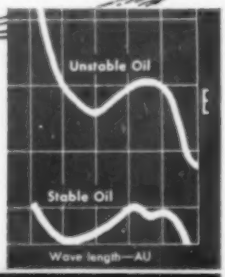

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Form of Presentation.—Titles of manuscripts should be kept as short as possible and preferably restricted to one line. The name of the laboratory in which the work described was done should be placed in a footnote at the end of the first page. Each manuscript should begin with one or more introductory paragraphs indicating the intention and meaning of the investigation and the method of procedure. The introduction should be followed by a report of the essential details of the experimental work, and a discussion or interpretation where necessary. Authors may find it helpful to arrange manuscripts to conform to the style of presentation as illustrated in papers on analogous subjects published in recent issues of THIS JOURNAL.

In preparing manuscripts for publication in THIS JOURNAL, center headings should be avoided as far as possible. Side headings followed by a period and dash should be indented and run into the part of the text to which they apply. Such side headings may be used as freely as desired. Literature citations should be numbered consecutively in order of appearance in the manuscript and should be indicated by full-sized Arabic numerals enclosed in parentheses. If a literature reference is cited more than once in a manuscript, subsequent citations should be indicated by the first number assigned. References to footnotes throughout the text should be numbered consecutively by superscript Arabic numerals, but such references in each table should be designated by superscript lower case letters beginning with "a" and continuing in natural order. Literature citations should be grouped at the end of the manuscript under the heading "References." All bibliographic references should be checked with the original publications. The citations should be numbered consecutively with full-sized Arabic numerals enclosed in parentheses corresponding to the numbers used in the text. The names of all periodicals cited in the list of references must be abbreviated in accordance with abbreviations given by **Chemical Abstracts** in its "List of Periodicals Abstracted." The sequence followed in the citations must be as follows:

(1) Doe, J. B., *Am. J. Physiol.*, **79**, 289(1927).

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(1) Gilman, H., "Organic Chemistry," Vol. 2, 2nd ed., John Wiley and Sons, New York, 1943, pp. 1236, 1252.

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Figures.—All drawings should be made in India ink, preferably on white tracing paper or cloth. Original drawings are preferable, but clear, well-prepared glossy photographic prints are acceptable. The size of illustrations should not exceed the 8.5″ x 11″ manuscript size. They should be designed for reduction to the width of one column (2.75″) or full page width (5.5″). Thus, height rather than width should be accentuated where possible.

Numbers and measurements, etc., which are to appear on the axes of the drawing should be indicated in pencil outside the axes, in order that they may be set in type. Captions and legends should be placed *below* the diagram (not on it), or, if lengthy, collected on a separate typewritten list, identifying each by its proper figure number and including whatever acknowledgments are necessary.

Photographs should never be rolled, and paper clips should not be used unless guarded by a piece of paper folded several times to prevent an indentation in the photograph. All drawings and photographs should be protected from bending and from the chance of having their edges torn while in transit. Do not write on the back of a photograph. Any indentation in the photograph, however slight, will be likely to show as a blemish in the reproduction. All figures submitted must be referred to in the text of the manuscript, and should be numbered consecutively with Arabic numerals, e. g., Fig. 1, Fig. 2, etc. Kymograph tracings should not be submitted with the original experimental legends which are likely to have been hastily or carelessly inscribed. Authors may find it worth while to employ a professional draftsman to make the drawings in proper form for reproduction.

Tables.—Tables should be carefully constructed so that the data presented may be understood easily. The indiscriminate use of tables should be avoided. Tables should be constructed so as to occupy fully a single column (2.75″) which will take about 40–50 characters and spaces. Tables which require two columns are also acceptable. Interpretative statements should not be written on the body of a table but should be explained in footnotes. Column heads should be made as small as possible and standard abbreviations should be used liberally. Data which may easily be deduced by simple arithmetic from data in another column should not be included. All tables should be referred to in the text of the manuscript. Every table and every column should be supplied with an appropriate heading. It is not necessary to place tables on separate sheets, and in fact it is preferable to intersperse them in the manuscript. Tables should be numbered consecutively using Roman numerals. The table number and the title should be placed in a continuous heading above the data presented. The preparation of a clear and concise table of data requires much planning and infinite care.

The United States Pharmacopeial Convention, Inc.

ANNUAL FINANCIAL STATEMENT

Based upon the Report of the Auditor for the year ended December 31, 1960

STATEMENT OF INCOME AND EXPENSE

Income:			
Sales of pharmacopeias:			
Collections		$230,374.00	
Add—Increase in accounts receivable		29,114.80	
			$259,488.80
Sales of reference standards:			
Collections		93,635.00	
Add—Increase in accounts receivable and adjustment of prior years' collections		6,430.81	
			100,065.81
Interest on investments, less amortization of bond premiums of $22.65			6,839.85
Interest on savings deposits			5,359.19
Use of text by others			120.00
Miscellaneous			158.48
			$372,032.13
Expense			
Revision		$118,048.12	
Less—Increase in inventory		5,273.81	
			$112,774.31
Administration			9,975.55
Publications		$145,536.95	
Less—Increase in inventory		87,714.00	
			57,822.95
Headquarters		$ 5,754.94	
Add—Depreciation expense			
Building	$2,430.77		
Furnishings and equipment	4,256.17	6,686.94	
			12,441.88
Convention			7,142.80
			$200,157.49
Excess of Income over Expense			$171,874.64

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT)

Receipts:	
Sales of pharmacopeias	$230,374.00
Sales of reference standards	93,635.00
Interest on investments	6,862.50
Interest on savings deposits	6,243.68
Use of text by others	120.00
Miscellaneous	158.48
	$337,393.66

Disbursements:	Revision	Administration	Publications	Headquarters	Convention	Furnishings and Equipment	Total
Printing and binding	...	...	$144,602.48	...	...	...	$144,602.48
Salaries	$ 54,701.64	$5,351.80	...	$1,890.30	$1,057.40	...	63,001.14
Honoraria[a]	25,800.00	...	...	...	...	...	25,800.00
Meetings	10,098.05	1,598.41	...	...	3,627.75	...	15,324.21
Supplies	2,936.18	230.02	...	...	968.83	...	4,135.03
Postage and telegrams	2,267.37	53.60	...	...	215.13	...	2,536.10
Utilities	...	...	...	2,711.36	...	...	2,711.36
Repairs and maintenance	...	...	...	743.33	...	...	743.33
General[b, c]	22,244.88[b]	2,741.72[c]	934.47	18.64	1,273.69	...	27,213.40
Insurance and taxes	...	...	...	391.31	...	...	391.31
Furnishings and equipment	...	...	...	...	...	$894.22	894.22
	$118,048.12	$9,975.55	$145,536.95	$5,754.94	$7,142.80	$894.22	$287,352.58

[a] Honoraria distributed to members of the U. S. P. Revision and related Committees for voluntary contributions in preparing U. S. P. XVI during the years 1956–1960.

[b] Reference Standards supplies	$ 8,531.92	[c] Counsel	$2,062.22
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JOURNAL OF Pharmaceutical Sciences

July 1961 volume 50, number 7

Review Article

Pharmacology of Antihypertensive Compounds

By JOSEPH P. BUCKLEY

HYPERTENSIVE CARDIOVASCULAR DISEASE

IT IS DIFFICULT to discuss the actions and uses of drugs utilized in the treatment of a disease when we do not fully understand the physiological disturbances producing the characteristics identifying the particular pathological condition. Unfortunately, this is true of hypertensive cardiovascular disease, a condition which is extremely widespread in the United States. The following definition of hypertension proposed by Goldring in 1944 (1) was suggested as a satisfactory definition of the hypertensive process at the Conference on Methodology in Epidemiological Studies of Cardiovascular Disease at Princeton, N. J., April 1959 (2): "The term hypertensive disease is synonymous with essential hypertension and should properly be restricted to designate the as yet unidentified physiological disturbance (or disturbances) characteristic of this disease and which leads ultimately to elevation of diastolic and systolic blood pressures, anatomical changes in the vascular tree, and functional impairment of the involved tissues."

There is increasing evidence that the stresses and tensions of daily living play a very important role in the development and progression of essential hypertension. The increase of arterial pressure with age is produced mainly by environmental factors operating in the population at large (3). High blood pressure appears to be directly related to the intensity and duration of environmental factors and is irrespective of age (3). Several authors have reported the production of hypertension in rats by subjecting the animals to daily audiogenic stimulation (4–6). Hypertensive cats have been produced by exposing the cats to aggressive dogs (7). Sustained hypertension in rats has been produced by subjecting the rats to daily discontinuous decompression for a period of eighteen weeks (8), and hypertensive rats have been prepared by subjecting them to experimental stress conditions for periods of eighteen to twenty-four weeks (9). Goldberger (10) has theorized that the pathogenesis of essential hypertension is psychogenic and that hypertension develops as a direct result of a psychologic factor, producing both immediate and delayed changes. The immediate changes are generalized sympathetic stimulation with a subsequent release of norepinephrine, producing increased peripheral resistance and hypertension and localized cholinergic stimulation of vasodilator nerves in specific skeletal muscles, followed by a further secretion of norepinephrine and aldosterone. The delayed changes are observed only if aldosterone secretion continues for a prolonged period of time. The aldosterone then causes a shift of intracellular body water into the extracellular spaces, and potassium in the cells is replaced by sodium and water. This produces a thickening of the intima and a decrease in the lumen size of the vessels, thus further increasing peripheral resistance and blood pressure. Urinary aldosterone secretion in patients with advanced essential and malignant hypertension has been reported to be twice that of normal individuals (11). Although it has not been definitely established whether aldosterone hypersecretion is a cause or result of malignant hypertension, the vascular lesions of malignant hypertension suggest that aldosteronism is a possible cause (12).

Received from the Department of Pharmacology, University of Pittsburgh, School of Pharmacy, Pittsburgh, Pa.

Russian investigators (13) have maintained that essential hypertension in dogs is a specific manifestation of neurosis and have produced experimental hypertension in dogs and monkeys by subjecting them to stress stimuli using Pavlovian techniques. If the primary cause of essential hypertension is neurogenic or psychologic due to the inability of the individual to adapt himself to stress situations, then those compounds which act centrally as depressants or ataractics should play an extremely important role in controlling the early stages of essential hypertension and perhaps preventing the progression of the disease.

The presence of humoral pressor agents in the blood stream may possibly be the cause of essential hypertension or the cause of the sustained elevated blood pressure in hypertensive patients. Since Goldblatt (14) successfully produced sustained hypertension in dogs by partially constricting both renal arteries, investigators have been trying to isolate pressor substances in the blood of hypertensive animals and patients. Renin, a protein-like substance extracted from normal kidneys, was found to be highly active when injected into intact animals but did not produce vasoconstriction when profused through a dog's tail or a rabbit's ear (15). The incubation of a mixture of renin and the renin-activator substance obtained from blood plasma resulted in a heat-stable, vasoconstricting pressor substance (angiotensin) (16). The renin-activator substance was found to be associated with the α-2-globulin fraction of hog serum (17). The crystalline pressor substance (angiotensin II) has been synthesized (18) and provides investigators with a very important tool for research into the mechanisms of essential hypertension. Angiotensin II is an octapeptide which appears to act directly on the smooth musculature of the vascular system (18–21). Recent studies (22) indicate that angiotensin II also acts through central hypertensive mechanisms, probably by stimulating the central sympathetic structures, thus evoking peripheral sympathetic discharges which can be blocked by the administration of piperoxan into the peripheral circulation.

The increase of aldosterone secretion by psychologic stress (10) suggests that this hormone may possibly be an important contributory factor in essential hypertension. Certain physiological reactions, which are important in the maintenance of homeostasis, have been shown to produce hyperaldosteronism. Constriction of the inferior vena cava (23), bilateral constriction of the common carotid arteries (24), and blood loss (25) are all adequate stimuli to increase aldosterone secretion. It has been postulated that a diminished intracarotid pulse pressure leads to an increase in aldosterone secretion by reducing the impulses in nerves arising at the thyrocarotid arterial junction, thus releasing inhibition of a central center which may control the release of a neurohormone thought to mediate increases in aldosterone secretion (24).

Although the exact physiological processes in essential hypertension are unknown, we actually know a great deal regarding this pathological condition. A large number of pressor substances have been isolated from the blood of hypertensive patients and animals (26), and one or more of these substances may be the responsible agent for the sustained elevated blood pressure in hypertensive patients. The question as to whether essential hypertension is renal in origin is debatable; however, there certainly is an increasing amount of evidence supporting the view that the kidney plays an ever important role in the development of hypertensive vascular disease (26). It is also possible that environmental factors may trigger the hypertension through stimulation of sympathetic centers in the subcortex thereby evoking sympathetic discharges in the periphery. It appears that one of the main causes of increased blood pressure in hypertensive patients is increased peripheral resistance (27, 28) with a concomitant increased sensitivity to the pressor effects of norepinephrine (27). It has also been suggested that essential hypertension is not due to generalized arteriolar spasm but caused by an inadequate vasodilator response of arterioles to increasing cardiac output (29).

Although there appears to be some disagreement on the pathogenesis of hypertension, it is generally felt that the proper use of hypotensive agents will prolong the life of the hypertensive patient (29–32).

THERAPEUTIC AGENTS

Centrally Acting Compounds.—The hypotensive agents which act on the central nervous system can be subdivided into those acting on the cerebral cortex and those acting at subcortical levels. The barbiturates depress the hyperexcitability of the cerebral cortex thus preventing impulses from reaching the sympathetic centers in the subcortex (33). The barbiturates depress the utilization of oxygen by the brain cells and inhibit the inorganic phosphate uptake of the cells thereby preventing the synthesis of compounds containing high energy phosphate bonds (34). There also appears to be a very close correlation between the increase in brain serotonin concentration and the depression produced by phenobarbital and other barbiturates (35) as well

as the anticonvulsant properties of phenobarbital and other anticonvulsants and the increase in brain serotonin concentration.

The compounds which act on the subcortical centers include the rauwolfia alkaloids and such ataractics as the phenothiazines and diphenylmethanes. The pharmacological and biochemical actions of reserpine on various parts of the body are complex and varied. Reserpine releases serotonin from its binding sites (36) and impairs the capacity of storage depots in brain cells to take up serotonin (37). This serotonin releasing activity is limited to those rauwolfia alkaloids possessing tranquilizing and antihypertensive activity, namely, reserpine, deserpidine, and rescinnamine (38). It has also been demonstrated that reserpine releases norepinephrine from the brain (39) and that the concentration of dopamine, a precursor to norepinephrine (40, 41), also decreases in the brain after reserpine administration (42). It appears that reserpine induces central inhibition of those afferent impulses which normally stimulate sympathetic activity within the central nervous system (43) and that reserpine exerts an action on the central mechanisms regulating circulation (44). Reserpine also inhibits the pressor response to central vagal stimulation and bilateral carotid occlusion; however, the pressor activity of norepinephrine is potentiated (45, 46). Since reserpine also releases norepinephrine from peripheral tissue as well as the brain (47), there is the possibility that the cardiovascular responses to reserpine might be due, in part, to the loss of the transmitter substance at peripheral nerve endings (48). Reserpine has been reported to decrease the norepinephrine content of the heart (49), the blood vessels (50), and the catechol amine concentration of the adrenal medulla (51). It has been suggested that reserpine acts as a hypotensive agent purely through its peripheral actions and not through its central activities (48); however, it would appear that in the dosage used in human patients the compound acts both through central and peripheral mechanisms. There is a possibility that reserpine increases parasympathetic discharges from the central nervous system rather than depressing sympathetic output (52). The side effects usually associated with the use of reserpine in hypertensive patients are characteristic of parasympathetic innervation, namely: increased nasal secretions, increased gastrointestinal activity, bradycardia, ocular disturbances, etc. The available data indicate that reserpine most likely acts through three specific mechanisms: (*a*) depletion of norepinephrine from its peripheral binding sites thus producing sympathetic blockade; (*b*) depression of central sympathetic outflow and/or stimulation of parasympathetic central outflow; (*c*) central nervous system depression producing a decrease in tension and aggressiveness and a decrease in the response of the sympathetic centers to external stimuli.

Since reserpine often produces severe central depression when administered over a prolonged period of time to hypertensive patients (53, 54), attempts have been made to synthesize derivatives of reserpine which might possibly be void of the central depressant activity. Syrosingopine, a chemical derivative of reserpine (55), has been reported to possess hypotensive properties in nondepressant doses (56). Since syrosingopine releases serotonin and catechol amines from peripheral binding sites (57), it has been postulated that the compound acts as a peripheral sympathetic blocking agent (48). Syrosingopine also lowers blood pressure through central mechanisms (58), and it appears that the mechanism of action of syrosingopine is identical to reserpine. Since the range of dosage over which syrosingopine depletes heart norepinephrine without the production of sedation is much wider than that with reserpine, it would be expected that syrosingopine should lower blood pressure in hypertensive patients and produce less of a depressant effect than reserpine (48).

GANGLIONIC BLOCKING AGENTS

Bisquaternary Ammonium Compounds.—Tetraethylammonium chloride has been reported to have a "nicotinic paralyzing" action (59) and is found to lower blood pressure through ganglionic blockade (60). Pentamethonium and hexamethonium lower blood pressure through a specific paralysis of transmission at ganglionic synapses and not on postganglionic fibers (61). They block nerve impulses at autonomic ganglia by raising the threshold of the ganglion cells to acetylcholine released at the preganglionic nerve endings (62). The bisquaternary ammonium compounds (hexamethonium, pentolinium, chlorisondamine, trimethidinium) are unable to excite autonomic ganglia but compete with acetylcholine for postganglionic receptor sites (63). Since this antagonism is competitive, the intensity of the block increases as the concentration of the bisquaternary ammonium compound in the extracellular space of the ganglion increases in relation to the acetylcholine release (62). There is inhibition of all autonomic ganglia because the compounds do not distinguish in their blocking activity between the sympathetic and parasympathetic systems (62).

The usefulness of these compounds is limited

partially because of the diminished parasympathetic outflow producing the following characteristic side effects of ganglionic blockade: dryness of the nose, mouth, and throat; constipation; impaired visual accommodation; and urinary retention (64, 65). The severity of the side effects usually can be decreased by administering a mild parasympathomimetic drug such as neostigmine (65). Since the bisquaternary ammonium compounds are very poorly absorbed from the gastrointestinal tract (66), there is difficulty in maintaining a constant blood level which further increases the possibility of side effects.

Trimethidinium (67) has been suggested to have a dual mechanism of action namely through ganglionic blockade and some form of centric depression (68). Cross circulation studies in dogs indicate that the compound does not possess any central hypotensive activity and that the drop in blood pressure is most probably due to ganglionic blockade (69). Clinical studies with trimethidinium also indicate that the compound acts mainly through ganglionic blockade, and the side effects of the compound are essentially due to this mechanism of action (70).

The bisquaternary ammonium compounds potentiate the pressor activity of various exogenous pressor agents including angiotensin, epinephrine, and norepinephrine (71). There appears to be a difference in the potentiation of the pressor activity of angiotensin and that of the pressor amines indicating that the potentiation cannot be ascribed simply to the blockade of compensatory autonomic reflexes or to the considerable increase in heart rate (72).

Beta - dimethylaminoethyl - N - methylpipecolinate dimethylbromide (JB-591) has been reported to be a potent ganglioplegic hypotensive agent (73, 74). Recent studies have shown that the compound possesses a dual mechanism of action, ganglionic blockade and a central hypotensive mechanism (75). JB-591 also appears to relieve hypertensive symptoms prior to an actual lowering in blood pressure and produces a feeling of tranquility in the majority of patients. Doses of 250 mg. administered one-half hour prior to eating produced marked hypotensive effects in human hypertensive patients, mainly through ganglionic blockade. If a dosage of 125 mg. is administered three times a day, there is a gradual drop in blood pressure which reaches a maximum in two to six weeks without the side effects characteristic of typical ganglionic blocking agents (75).

Mecamylamine and Pempidine—Mecamylamine, a secondary amine, and pempidine, a tertiary amine, differ from the bisquaternary ammonium compounds in that their oral absorption is greater than 50 per cent (76). The chemical dissimilarity in structure between mecamylamine and the bisquaternary ammonium compounds suggests a different mechanism of action. There is a difference in the type of response of the nictitating membrane to preganglionic stimulation of the cervical sympathetic nerve with mecamylamine and the bisquaternary ammonium compounds (77). Both mecamylamine and pempidine have relatively long durations of action (76, 78). Mecamylamine and pempidine potentiate *d*-tubocurarine and antagonize decamethonium (76) and, in larger doses, produce neuromuscular blockade. The neuromuscular blockade induced by mecamylamine and decamethonium cannot be antagonized by neostigmine (77). The side effects of mecamylamine and pempidine are characteristic of ganglionic blocking agents and due to sympathetic and parasympathetic ganglion blockade (79). Mecamylamine also produces weakness and fatigue, probably due to the mild skeletal muscle relaxant action. There appears to be two main advantages of mecamylamine and pempidine over the bisquaternary ammonium compounds: (*a*) The greater degree of absorption and less variability of absorption prevents great fluctuations in blood pressure with less tendency for the production of severe postural hypotension; therefore, the hypotensive effects are more predictable than those obtained with the bisquaternary ammonium compounds. (*b*) Both compounds have a much longer duration of action than most of the bisquaternary ammonium compounds.

SYMPATHETIC BLOCKING AGENTS

Since there is the strong possibility that the elevated blood pressure in essential hypertension is due to increased sympathetic outflow and since it has been demonstrated that surgical interruption of this outflow (80) can lower blood pressure in the essential hypertensive patient, it would appear that compounds that block the release or inhibit the effect of the sympathetic mediator at the neuroeffector sites would be ideal hypotensive agents. Ergot has been known to block and reverse many of the responses to circulating epinephrine and sympathetic nerve stimulation (81), and it appears that certain ergot alkaloids interfere with sympathetic nerve transmission by blocking or diminishing the effect of myoneural hormones. Cannon has suggested that there are two sympathetic mediator substances, sympathin-E and sympathin-I (82). The sympathetic neurohormone contains a very high percentage of norepinephrine and a very

small amount of epinephrine (83) with norepinephrine corresponding to sympathin-E and epinephrine corresponding to sympathin-I (84). It has also been postulated that there are two types of adrenotropic receptors, alpha and beta (85), and that the alpha receptors are associated with most of the excitatory functions (vasoconstriction, nictitating membrane stimulation, etc.) and one inhibitory function (intestinal relaxation). The beta receptors are associated with most of the inhibitory functions (vasodilatation) and one excitatory function (myocardial stimulation). Epinephrine stimulates both the alpha and beta receptors, and the overall effect on blood pressure is a pressor response. *Levo*-norepinephrine stimulates only alpha receptors, thus producing pressor responses. Such compounds as ergotoxin block alpha receptors, and subsequent doses of epinephrine stimulate only the beta receptors producing vasodilatation and the typical hypotensive responses associated with epinephrine reversal. Since *l*-norepinephrine stimulates only the alpha receptors, ergotoxin either blocks the pressor effect or diminishes the response.

The beta-halo alkylamines (dibenamine and phenoxybenzamine) are both potent adrenergic blocking agents (86, 87). Although these compounds selectively block the sympathetic division and circulating epinephrine and norepinephrine and have been useful in the treatment of peripheral vascular disease, their use in the treatment of hypertension has been limited; and the clinical data obtained with them have not been too encouraging (88). This is probably due to the extremely potent blocking activity of the compounds producing postural hypotension and tachycardia and is most likely due to a firm binding with the receptor substance of the effector organ (89). The mechanism of action of phenoxybenzamine appears to be a competition with the catechol amines for the binding sites of receptor substances of the vascular smooth muscle cells. The use of phenoxybenzamine in the treatment of hypertension has been disappointing because of the numerous side effects, especially dizziness, due to reduced cerebral blood flow and tachycardia due to postural hypotension (90).

Recently, two compounds, guanethidine (91) and bretylium (92), have been reported to block peripheral sympathetic outflow selectively through mechanisms other than receptor site blockade.

Guanethidine.—Guanethidine is an orally effective sympathetic blocking agent which appears to act by depleting norepinephrine from peripheral nerve endings (93, 48). Intravenous administration of guanethidine to anesthetized normotensive dogs produces moderate hypertension persisting for periods of approximately forty-five minutes, which is then followed by a gradual progressive decline in the mean blood pressure. This dual response indicates a possible initial release of pressor amines (93). Guanethidine antagonizes the hypertension induced by large doses of amphetamine and bilateral carotid occlusion and produces a relaxation of the nictitating membrane in dogs and cats. The pupils are dilated and accommodation to light decreased. It reduces the catechol amine content of the rabbit and cat heart by approximately 80 per cent but does not lower the amine content of the brain or adrenal medulla (94), which suggests that the compound lowers blood pressure by producing chemical sympathectomy through the depletion of norepinephrine from peripheral nerve endings. Intravenous administration of guanethidine to hypertensive patients produced an initial hypertensive response (95). It potentiates the pressor response to angiotensin, vasopressin, metaraminol (96), epinephrine, Synephrine, and phenylephrine (97). The compound has been reported to lower blood pressure by reducing cardiac output rather than by relaxing the arterioles and thereby reducing peripheral resistance (98, 99). Guanethidine appears to be a reasonably effective antihypertensive agent having minimal side effects (100); and from the available data, it appears to be most effective in the treatment of moderate to severe hypertension. The available pharmacological data suggest that the compound should be used with extreme care since it does potentiate certain pressor amines which might be found in the hypertensive patient in relatively high quantities or which might possibly be administered for the treatment of other diseases. It is also apparent that if the compound is administered to a person suffering from a pheochromocytoma, severe hypertensive reactions might occur. Although most of the available literature indicate that guanethidine acts entirely through peripheral mechanisms, there has been a report that some of the hypotensive activity may either be central in nature or due to the stimulation of certain central hypotensive reflexes (101).

Bretylium.—Bretylium tosylate is a benzyl quaternary ammonium compound which selectively blocks the sympathetic nervous system without affecting the parasympathetic division (92). The compound accumulates selectively in adrenergic nerves in sufficient concentrations to impair their conduction. In the presence of

bretylium, the stimulation of certain sympathetic nerves fails to release norepinephrine; however, the effects of circulating epinephrine and norepinephrine are potentiated. Sympathetic nerve trunks and sensory nerves in the skin are readily blocked for long periods by the topical application of bretylium, indicating a local anesthetic activity. The mechanism of action does not appear to be due to a peripheral depletion of norepinephrine or an inhibition of the synthesis of norepinephrine (48) but possibly to the prevention of the release of the sympathetic neurohormone (92). Concentrations of bretylium capable of inducing sympathetic blockade do not prevent the release of epinephrine and norepinephrine from the adrenal medulla by splanchnic nerve stimulation (92). The compound produces a decrease in cardiac output (102, 103). High concentrations of bretylium induce a curare-like paralysis (92), and muscle weakness or fatigue has been reported in patients treated with this hypotensive agent (104). Bretylium has been reported to have produced parotid pain which apparently is not due to obstruction of Stensen's ducts (105). Since the drug is a quaternary ammonium compound, it is poorly and irregularly absorbed (105, 106); and this variability in absorption may lead to either severe postural hypotension or a poor hypotensive response. The available data indicate that bretylium is a potent hypotensive agent which interferes with sympathetic nerve function without affecting the parasympathetic division. The major disadvantages of the compound appear to be its erratic and poor absorption and its potentiation of the pressor effects of epinephrine and norepinephrine. A severe hypertensive crisis may occur if bretylium is administered to a patient suffering with a pheochromocytoma (106). Additional side effects reported with the compound are postural hypotension, parotid pain, tiredness, and muscle fatigue, and the development of tolerance to the compound.

COMPOUNDS DEPRESSING VASCULAR SMOOTH MUSCULATURE

Certain inorganic nitrites, and organic nitrites, and nitrates lower blood pressure by directly depressing vascular smooth muscle (107). Although their exact mechanism of action is not completely understood, some evidence has been presented that these compounds act at the enzyme level. Certain of these compounds have been shown to affect the adenosine triphosphate-adenosine triphosphatase system (108, 109) by interfering with adenosine triphosphatase activity of arterial tissue. It has been suggested that arterial tone and constriction is maintained by the decomposition of ATP by its enzyme, and that any interference with ATPase activity would produce relaxation of the artery. Sodium nitrite does not directly affect the ATP-ATPase system (109); however, it inhibits oxygen uptake of arterial tissue which is concerned with resynthesis of ATP. Although there has been some evidence that the vasodilating property of organic nitrates is due to their conversion to nitrites (110), it now appears that the organic nitrates produce a reduction in the blood pressure by virtue of their own molecular structure and not through hydrolysis and reduction to nitrite (107). Mannitol hexanitrate inhibits phosphorylation in rat liver mitochondria (111). Mannitol hexanitrate and erythrityl tetranitrate are utilized as hypotensive agents; however, they have been disappointing in the treatment of hypertension (107). Both compounds are slowly absorbed from the intestinal tract and rapidly excreted (112). The nitrites and nitrates have produced undesirable side effects such as headache, throbbing in the temples, weakness, fainting, etc. Several of the organic nitrates (glyceryl trinitrate, pentaerythrityl tetranitrate, etc.) have proved to be extremely valuable in the treatment of angina pectoris (113–115).

Hydralazine.—Hydralazine is a potent hypotensive agent (116) which simultaneously dilates peripheral blood vessels, increases cardiac output (117), and markedly increases renal blood flow which is not accompanied by increase in glomerular filtration rate (118, 119). The pharmacology of hydralazine is rather complex and the exact mechanism of the hypotensive activity is not clearly understood. The compound appears to act through both central and peripheral mechanisms. Hydralazine was first believed to act at either cortical or subcortical sites, as it was found to be inactive when administered to spinal cats and dogs (116, 120). It inhibits the pressor substance elaborated by the central nervous system (121) and other humoral pressor agents such as angiotensin (122, 123), pherentasin (122, 123), serotonin (121), norepinephrine (123), epinephrine (124), and tyramine (123). Hydralazine inhibits DOPA decarboxylase (125) and may therefore inhibit norepinephrine biosynthesis, producing a blockade of the sympathetic division. It chelates certain trace metals, especially copper (126), and blocks copper induced constriction of coronary vessels. The primary action of hydralazine appears to be on some basic mechanism in the vascular smooth muscle cell concerned with vasoconstriction (125), thus the compound ap-

pears to resemble the nitrites and nitrates in its basic mechanism of action. Unfortunately, hydralazine has produced a large number of side effects in the human hypertensive patient, and these have limited its usefulness as an antihypertensive compound. The compound has been reported to produce a syndrome of rheumatoid joint manifestation associated with headaches, fever, and prostration (127); lupus-like syndrome with the presence of L. E. cells in the peripheral blood of severe cases (128); edema of the eyelids (125); nasal stuffiness (125); headaches (129); nausea (129); and precordial pain (129). Chronic studies suggest that the administration of doses below 400 to 500 mg. per day will not produce severe toxic effects. It is most likely that the side effects of hydralazine can be decreased by administering the compound in combination with other antihypertensive agents (130, 131).

VERATRUM ALKALOIDS

Numerous hypotensive tertiary amine alkaloidal esters have been obtained from the roots of veratrum plants. A biologically standardized extract containing a mixture of the alkaloids of *Veratrum viride* has been reported to produce potent hypotensive responses (132–134). These compounds appear to act mainly through a reflex vasodepressor action (135, 136); however, there is no unanimity among investigators regarding the sites of action of the veratrum alkaloids inducing depressor responses. Although these compounds stimulate certain afferent reflex pathways from the carotid sinuses, aortic arch, and pulmonary arteries (137, 138), the main mechanism of action appears to be through the Bezold-Jarisch reflex in which the afferent fibers arise in the walls of the coronary arteries in the left ventricle (139, 140). The Bezold-Jarisch reflex is not blocked by carotid denervation (141, 142) but is completely blocked by bilateral vagotomy (142). The veratrum alkaloids also block the carotid sinus pressor reflex (135), and this physiological response has been used as a method for assaying the potency of *Veratrum viride* (143, 144). These compounds have also been reported to have a direct effect on central structures (145). Veriloid has been reported to lower blood pressure in dogs solely by decreasing cardiac output (146); however, a recent study on the cardiovascular effects of some germine esters suggests that the hypotension is due to a decline of both vascular tone and cardiac output (147). In addition to the hypotensive response, the main physiological effects produced by the veratrum alkaloids are bradycardia (148) and emesis (149). The production of nausea and emesis in the majority of patients has limited the use of the veratrum alkaloids in the treatment of hypertension. Since the margin between the effective hypotensive dose and effective emetic dose is very narrow, the dosage in any particular patient should be titrated in the same manner in which diabetes is treated by titrating the dose of insulin and the dosage of cardiac glycosides determined in the treatment of congestive heart failure (150).

DIURETICS

The importance of electrolyte concentration and electrolyte balance in the production and control of hypertension has been known for many years, and the use of low salt diets or salt-free diets has been recommended for the treatment of arterial hypertension (151, 152). It appears that the balance between sodium and potassium may play an important role in maintaining arterial tone since sodium possesses vasoconstrictor properties and potassium vasodilator properties (153). The rice diet which has been employed in the treatment of hypertension is essentially a low sodium, high potassium diet (154). Diuretics have been shown to possess hypotensive activity and to potentiate the depressor effects produced by antihypertensive compounds (155–159). The use of diuretics in the treatment of essential hypertension was extremely limited until the introduction of chlorothiazide (160, 161). The oral administration of chlorothiazide lowers the blood pressure in arterial hypertension and markedly potentiates other hypotensive agents (160–162). The mechanism of action of chlorothiazide and other benzothiadiazines on the circulation is not clearly understood (163); however, the blood pressure lowering properties of chlorothiazide has been reported to be selective to hypertensive patients (159). It appears that the mechanism of the hypotensive activity of the benzothiadiazine derivatives is mainly due to the elimination of sodium (164–166), which results in a reduction in both interstitial fluid and plasma volume (159, 167). Although some investigators have suggested that the diuretics may lower blood pressure by interfering with some arterial pressor mechanism (168), it appears that the reduction in blood pressure is due to a decrease in plasma volume (166). Since sodium is a vasoconstrictor substance (169), it would appear that a drop in sodium concentration should produce a dilatation of the vascular system. Chlorothiazide also decreases cardiac output and inhibits the pressor response to norepinephrine in hyper-

tensive patients (163) and may decrease the production of renin (170). Chlorothiazide has also been reported to decrease total vascular resistance (171).

Compounds which antagonize the renal tubular action of aldosterone and related mineral corticoids by competitive inhibition have recently been introduced (172–176). Spironolactone, an aldosterone antagonist, is an orally effective diuretic which acts through different mechanisms than those of other compounds currently in use, making it possible to act synergistically with other diuretics (175, 176). The compound also differs from other diuretics in that although there is an increase in sodium excretion, there usually is no change or an actual decrease in potassium excretion (174, 175). Spironolactone does have an antihypertensive effect on hypertensive rats (177). If essential hypertension associated with stress involves an increased production of aldosterone, it would appear that the antialdosterone compounds might play an important role in the control of hypertension.

The orally effective diuretics, especially the benzothiadiazines and antialdosterone compounds, appear to be extremely useful in the management of arterial hypertension (168). The diuretics are of particular interest in that they do potentiate the hypotensive activity of other antihypertensive compounds, thus lower dosages of potent hypotensive agents can be utilized when combination therapy is used (178).

ENZYME INHIBITORS

Two groups of enzyme inhibitors possess antihypertensive properties, the monoamine oxidase inhibitors and the decarboxylase inhibitors.

Monoamine Oxidase Inhibitors.—Iproniazid was reported to be a potent *in vitro* inhibitor of monoamine oxidase (179) and was later found to be a potent inhibitor of monoamine oxidase *in vivo* (180, 181). Beta-phenylisopropylhydrazine also inhibits monoamine oxidase (182) and produces a rapid elevation in brain serotonin and a slower rise in brain norepinephrine (183). Although the compound produces pressor responses in dogs, it was also found to be a potent hypotensive agent in human hypertensive patients (184, 185). This effect is apparently not exclusive with β-phenylisopropylhydrazine as the administration of a large number of monoamine oxidase inhibitors have also produced a decrease in blood pressure and a marked increase in urinary tryptamine (186). Available data indicate that these compounds are synaptic blocking agents (187–191) and that the mechanism of the hypotensive activity is most likely ganglionic blockade (191). It has been suggested that norepinephrine is antagonistic to the acetylcholine effect in sympathetic ganglia (192) and that since monoamine oxidase inhibitors increase the concentration of norepinephrine, an increased concentration of norepinephrine in autonomic ganglia would produce a blockade of ganglionic transmission (48). Since these compounds mainly produce a postural-type hypotension (185) and have been reported to be toxic to the liver (193), their use as antihypertensive agents has been extremely limited. At present, the potential toxicity of these compounds does not warrant their usage by the general practitioner for the treatment of essential hypertension.

Decarboxylase Inhibitors.—Derivatives of phenylalanine have been reported to inhibit the decarboxylation of dihydroxyphenylalanine (194). Alpha-methyl-dopa has been reported to be a potent decarboxylase inhibitor (194) preventing the decarboxylation of DOPA to dopamine (186), thus inhibiting norepinephrine synthesis. The compound has also been shown to inhibit 5-HTP decarboxylase, thus preventing the synthesis of serotonin from 5-hydroxytryptophan which decreases brain serotonin levels (195–197). Alpha-methyl-dopa has produced marked hypotensive effects in hypertensive patients both in the recumbent and standing positions (198). Since norepinephrine is the sympathetic neurohormone, it has been speculated that the consistent reduction of standing blood pressure by α-methyl-dopa is due to a reduction in norepinephrine biosynthesis (198). Recent studies, however, suggest that the pharmacological effects of α-methyl-dopa in the dog do not appear to be related to the decreased function of the peripheral sympathetic nerves or to an adrenergic blocking action (197); and there is the possibility of the existence of some other mechanism of action unrelated to decarboxylase inhibition. The compound also appeared to produce some degree of tranquility or central nervous system depression, indicating a possible central mechanism as well as the peripheral actions (198).

Since the pharmacological data on the decarboxylase inhibitors are still limited, it is difficult to evaluate the potentiality of these compounds as hypotensive agents. The available information indicates that the decarboxylase inhibitors may play an important role in the treatment of essential hypertension and that further pharmacological and clinical studies are warranted.

SUMMARY AND CONCLUSION

During the past decade, investigators have made great strides in their search for useful hypotensive agents. Unfortunately, all of the therapeutic agents in current use have major side effects which limit their usefulness. While the diuretics appear to be the most desirable compounds, they have been reported to produce hypokalemia (158, 199), skin rash (200), increased serum uric acid levels (201, 202), and other toxic reactions. The use of two or more hypotensive agents in combined therapy appears to be the best way of treating moderate to severe hypertension. The diuretics apparently potentiate the hypotensive activity of many antihypertensive agents, suggesting that very low dosages might be utilized to control the elevated blood pressure, thereby decreasing the possibility of severe side effects occurring.

It is apparent that the real breakthrough will not occur until we fully understand the underlying mechanisms of essential hypertension. The possible roles of norepinephrine, angiotensin pherentasin, serotonin, and aldosterone in the maintenance of elevated blood pressure are not clear. The influence of serotonin on the cardiovascular system is especially confusing since it is capable of decreasing or increasing the blood pressure when administered i. v. to humans (203). Rauwolfia alkaloids, which lower central and peripheral concentrations of serotonin and norepinephrine, lower blood pressure and induce central depression. The barbiturates also lower blood pressure and produce central depression, yet they apparently increase the concentration of serotonin in the central nervous system. The monoamine oxidase inhibitors increase the concentration of serotonin and norepinephrine, producing both hypotensive effects and central stimulation. A serotonin inhibitor (1-benzyl-2 - methyl - 5 - methoxytryptamine, BAS) lowers blood pressure in experimental animals and in patients by competitively inhibiting serotonin (204). Clinical studies have shown BAS to be both mildly antihypertensive and moderately depressant (205). Although it has been postulated that serotonin and norepinephrine are centrally antagonistic to one another (206), other investigators have demonstrated that the compounds block cerebral synaptic transmission (207, 208). The administration of serotonin, norepinephrine, and reserpine into a cerebral lateral ventricle of dogs produce hypotension and bradycardia (209). The decarboxylase inhibitors appear to partially block the synthesis of both norepinephrine and serotonin and may have a direct action of their own on the cardiovascular system. The data on serotonin are certainly conflicting and somewhat confusing; however, they suggest some relationship between alterations in body chemistry in essential hypertension and mental disease. Animals subjected to experimental stress demonstrate a marked increase in blood pressure and changes in behavioral patterns (9). Cardiovascular and psychopharmacological research should be coordinated whenever possible and not studied as separate entities. The exact roles of serotonin and the humoral pressor agents in essential hypertension must be elucidated before a more intelligent approach to research and therapy can be initiated. The results obtained thus far with competitive inhibitors have been encouraging and suggest that research should be expanded in this general area. Since angiotensin II is an octapeptide, research on hypotensive polypeptides should be increased with special attention placed on mechanisms of action.

Although relatively little space has been devoted to the veratrum alkaloids, their usefulness in the treatment of essential hypertension could be greatly expanded if there was a better understanding of these compounds, both from the pharmacological and clinical viewpoints. It is this author's opinion that if the veratrum alkaloids were the only hypotensive agents available for the treatment of essential hypertension they would be as useful as the digitalis glycosides are in the treatment of congestive heart failure. Research on these compounds should be expanded, and a greater variety of dosage forms should be made available to the clinical investigator so that the precise dosage on an individual basis can be prescribed and human pharmacology studied more intensely.

Compounds have been introduced recently which lower blood pressure through new and extremely interesting mechanisms. The effects of the sympathetic division on the cardiovascular system can now be diminished by competitive inhibition of the neurohormone (phenoxybenzamine), by ganglionic blockade (mecamylamine), by the depletion of the neurohormone from peripheral sites (guanethidine, reserpine), by the inhibition of the release of the neurohormone (bretylium), by the inhibition of the synthesis of the neurohormone (α-methyl-dopa), and by central inhibition (reserpine). The introduction of each new type of drug adds greatly to our understanding of the basic physiological processes of essential hypertension.

The search for new and better therapeutic agents will continue as will the search for the

underlying mechanisms of cardiovascular hypertensive disease. With each bit of added knowledge opening new avenues for research, essential hypertension will be treated more scientifically with true antihypertensive agents which will be safer and more effective, thereby permitting the patient to continue to live a "normal" productive life.

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Analysis of Conjugated Estrogen Preparations

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A method is proposed for the determination of estrone, equilin, and total estrogens in commercial conjugated estrogen preparations. The quantitative procedure employs a simple column separation and direct colorimetric estimations. Identity is established by paper chromatography. Assays of typical preparations are reported.

WATER-SOLUBLE conjugated estrogens are widely used for the oral treatment of certain hormone deficiencies. The principal source of this material is equine pregnancy urine, in which the steroids occur as complex mixtures of esters. Many analytical procedures have been proposed for these preparations (1). The relatively simple procedures estimate total estrogens colorimetrically using estrone as a standard. These analyses give no indication of the relative concentration of the individual hormones. Those methods designed to determine each of the physiologically active components require many operations and are, consequently, time consuming (2). Analysis of a large number of conjugated estrogen samples has shown that estrone and equilin constitute the major portion of the therapeutically active material present. A method capable of determining these two steroids directly, without involved separations, would be entirely satisfactory for the standardization of these drugs.

Beall and Grant (3) isolated water-soluble conjugated estrogens by means of a chloroform-soluble complex with dicyclohexylamine. Troublesome emulsions are frequently encountered when this procedure is applied to powdered tablet samples using conventional liquid-liquid extraction techniques. The proposed method, in a single operation, employs this reagent (as the acetate) and a water-siliceous earth column to separate the conjugates from "free" estrogens and diluents or tablet excipients. No emulsions are encountered. After this separation, hydrolysis is effected by the alcohol-acid method of Sanders, *et al.* (4). The principal estrogens are identified by a simple and rapid ascending paper chromatogram using the technique of Fischbach and Levine (5). Total estrogens are determined colorimetrically by the iron-Kober procedure using an estrone standard. The color produced by equilin is about one-half the intensity of that produced by estrone in this reaction, and the total estrogen content is accordingly corrected by the addition of one-half the quantity of equilin subsequently determined. The ketosteroid fraction of an aliquot of the hydrolysate is isolated by means of the Girard reaction (6), and estrone and equilin are determined by new modifications of the Kober reaction.

Estrone is routinely determined by the iron-Kober reagent first proposed by Haenni (7). In this reaction equilin and equilenin also produce significant amounts of color. Recent work in this laboratory has shown that a heating time of ninety minutes is necessary to form a reproducible color with estrone. When the heating time is increased to two hours no loss of estrone color occurs, and the colors formed with equilin and equilenin are minimized (Fig. 1*A*). In the proposed method for estrone a suitable correction is made for the equilin content. The small effect of equilenin is disregarded, since this steroid is always a minor component of natural estrogenic substances.

A previous report from this laboratory noted that equilin produced a color with a blue component when treated with Kober reagent (8), and that the intensity of blue was enhanced by the presence of alcohol. This chromogenic reaction is complex. In the absence of alcohol, the yellow color initially developed is rapidly transformed to blue with the simultaneous formation of a red component (Fig. 1*B*). In the proposed method, equilin is dissolved in 0.1 ml. of alcohol prior to the addition of iron-Kober reagent. Under these conditions the red component is not formed. Complete conversion to a stable blue color that follows Beer's law is effected by heating at 100° for twenty-five minutes. Estrone and equilenin offer negligible interference in this reaction (Fig. 1*C*).

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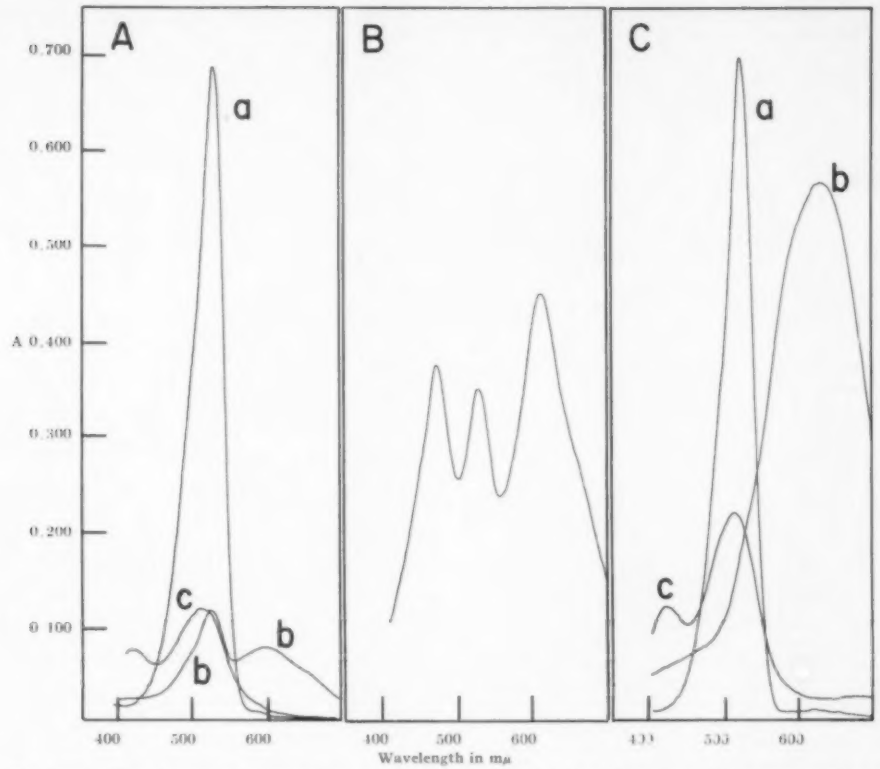

Fig. 1.—A. The absorption spectra of the colors produced by (*a*) 50 mcg. estrone, (*b*) 20 mcg. equilin, and (*c*) 50 mcg. equilenin, when treated with iron-Kober reagent with two-hour heating in the proposed procedure for estrone.

B. The absorption spectrum of the color produced with 50 mcg. equilin and iron-Kober reagent, fifteen-minute heating period.

C. The absorption spectra of the colors produced by (*a*) 50 mcg. estrone, (*b*) 20 mcg. equilin, and (*c*) 50 mcg. equilenin in the proposed procedure for equilin.

EXPERIMENTAL

Assay

Reagents.—Iron-Kober reagent. Iron-phenol reagent U. S. P. XVI, p. 277. Dicyclohexylamine acetate—dissolve 50 Gm. dicyclohexylamine in 150 ml. acetone, cool in an ice bath, and add, with stirring, 18 ml. acetic acid dissolved in 150 ml. acetone; filter the precipitate on a Büchner funnel, wash with a small amount of acetone, and air dry. Girard reagent T—trimethylacethydrazide ammonium chloride U. S. P. XVI, p. 1061.

Standard Preparation.—Dissolve about 5.0 mg., accurately weighed, of U. S. P. Reference Standard Estrone in benzene, warming if necessary. Cool and transfer to a 100-ml. volumetric flask and dilute to volume with benzene. Dissolve about 2.0 mg., accurately weighed, of purified equilin in benzene, warming if necessary. Cool and transfer to a 100-ml. volumetric flask and dilute to volume with benzene. (A U. S. P. reference standard grade of equilin may be available soon.)

Procedure.—In the case of tablet preparations, weigh 20 tablets and reduce to a fine powder without loss. Weigh a portion of the sample equivalent to about 7 mg. of sodium estrone sulfate and transfer to a 250-ml. beaker. For other preparations, use the equivalent of 7 mg. of sodium estrone sulfate. Add 6 Gm. of siliceous earth and mix thoroughly. Add 4 ml. water and mix until uniform. Transfer quantitatively, with the aid of a small amount of dry siliceous earth, to a 25 × 150 mm. chromatographic tube. Tamp moderately tight and wash the column with 100 ml. chloroform, discarding the chloroform wash. Add 100 mg. of dicyclohexylamine acetate dissolved in 5 ml. of chloroform to the column and collect the effluent in a 250-ml. glass-stoppered flask. Wash the column with several 5-ml. portions of chloroform and finally with sufficient chloroform to produce a total eluate of 150 ml. Evaporate the solution to dryness on a steam bath with the aid of a current of air.

Dissolve the residue in 25 ml. of absolute methanol. Add 1 ml. hydrochloric acid and a few boiling chips, stopper loosely, and boil for five minutes. Cool and transfer the solution to a 125-ml. separator with 70 ml. of water. Extract the aqueous solution with four successive 25-ml. portions of chloroform. Evaporate the combined chloroform extract just to dryness on a steam bath with the aid of a current

of air. Dissolve the residue in 5 ml. of chloroform and transfer, with the aid of not more than 5 ml. of chloroform, to a 125-ml. separator containing 50 ml. of isooctane. Add 10 ml. of sodium hydroxide solution (1 in 10) and shake for one minute. Transfer the aqueous layer to a second 125-ml. separator. Extract with two additional 10-ml. portions of sodium hydroxide solution, adding each to the second separator. Discard the chloroform-isooctane solution. Acidify the combined alkaline extract with dilute sulfuric acid, cool, add 25 ml. of benzene, and shake for one minute. Transfer the aqueous layer to a second separator containing 25 ml. of benzene, shake for one minute, and drain off and discard the aqueous layer. Wash each benzene fraction serially with 10 ml. sodium carbonate solution (1 in 10) and two 10-ml. portions of water. Filter the benzene layers through a pledget of cotton previously washed with benzene, into a 100-ml. volumetric flask. Wash the separators and funnel with benzene, filter the washings into the volumetric flask, make to volume, and mix (solution A).

Evaporate a 50-ml. aliquot of solution A to about 5 ml. and transfer to a 25-ml. glass-stoppered Erlenmeyer flask with the aid of a small volume of chloroform. Evaporate to dryness on a steam bath with the aid of a current of air. (Reserve the remainder of the solution A for the total estrogen determination and the paper chromatographic test for purity.) Add 100 mg. of Girard reagent T and 0.5 ml. of glacial acetic acid to the flask, stopper loosely, and heat on a steam bath for five minutes. Swirl the flask several times to ensure complete mixing. Cool the flask and transfer the contents, with about 25-ml. of ice water, to a 125-ml. separator. Add 5 ml. of sodium acetate solution (1 in 20) and extract immediately with three 10-ml. portions of chloroform. Combine the chloroform layers in a second separator, wash with 5 ml. of ice water, and discard the chloroform extract. Add the water wash to the aqueous solution in the first separator. Add 3 ml. of sulfuric acid solution (1 in 3), mix well, and allow to stand for thirty minutes. Extract the aqueous solution with three 25-ml. portions of chloroform. Combine the extracts in a second separator, wash with 5 ml. of water, and filter through a pledget of cotton previously washed with chloroform into a 150-ml. beaker. Wash the separator and filter with a small amount of chloroform and add to the main extract. Evaporate the chloroform solution to dryness on a steam bath with the aid of a current of air. Dissolve the residue in a small amount of benzene, warming if necessary, transfer quantitatively to a 50-ml. volumetric flask, and make to volume with benzene (solution B).

Determination of Total Estrogens as Sodium Estrone Sulfate.—Transfer duplicate 1-ml. aliquots of solution A and the estrone standard solution (using the same pipet) to 16 × 150 mm. glass-stoppered test tubes. Add a few boiling chips to each tube and suspend the tubes in a steam bath to initiate boiling. When boiling just ceases at the surface of the chips, remove the tubes from the bath and place them in a vacuum desiccator for thirty minutes. Do not permit the tubes to remain in the steam bath longer than necessary. To each tube and a blank tube, add 1.0 ml. of iron-Kober reagent, stopper, and place in a boiling water bath. After heating about one minute, shake the tubes to mix the contents, and release the pressure by momentarily removing the stopper. Heat for ninety minutes. Cool the tubes in a water bath and add to each 10.0 ml. sulfuric acid (1 in 3), and mix.

Record the absorbance of the sample and standard solutions, relative to the blank, between 400 and 700 mμ, and determine the baseline absorbance at 520 mμ, drawing the baseline between the minima at about 400 and 700 mμ.

Calculate the total estrogens by the formula

$$\text{sodium estrone sulfate mg./Gm.} = \frac{1.38 \times A_B \times C_1}{S_1 \times w} + \frac{d}{2}$$

where A_B = baseline absorbance of sample solution at 520 mμ, S_1 = baseline absorbance of standard solution at 520 mμ, C_1 = mg. estrone used in preparing the standard, w = weight of sample in Gm., d = mg. sodium equilin sulfate/Gm. as determined under sodium equilin sulfate, and 1.38 = the factor for converting estrone and equilin to their respective sodium sulfate esters.

Determination of Equilin as Sodium Equilin Sulfate—Transfer duplicate 1-ml.[1] portions of solution B and the equilin standard solution (using the same pipet) to 16 × 150 mm. glass-stoppered test tubes. Add a few boiling chips to each tube and evaporate as directed under determination of total estrogens. To each tube and a blank tube add 0.10 ml. of alcohol and rotate the tube to dissolve the ketosteroids. Add 1.0 ml. of iron-Kober reagent to each tube, mix thoroughly, stopper, and heat in boiling water bath for twenty-five minutes. Cool, add 3.0 ml. of sulfuric acid solution (1 in 3) to each tube, and mix thoroughly. Determine the absorbance of each solution, relative to the blank, at 620 mμ.

$$\text{sodium equilin sulfate mg./Gm.} = \frac{1.38 \times A_2 \times C_2}{S_2 \times w}$$

where A_2 = absorbance of sample solution at 620 mμ, S_2 = absorbance of equilin standard solution at 620 mμ, C_2 = mg. equilin used in preparing the standard, and w = wt. of sample in Gm.

Determination of Estrone as Sodium Estrone Sulfate.—Transfer duplicate 1-ml. aliquots of solution B, the estrone standard solution, and the equilin standard solution to 16 × 150 mm. glass-stoppered test tubes. Proceed as directed under the determination of total estrogens beginning, "add a few boiling chips ...," except heat for two hours instead of 90 minutes. Determine the absorbance of sample and standard solutions, relative to the blank, at 520 mμ. Correct the absorbance of the sample solution for the equilin absorbance as follows:

$$A_3\,(\text{corr.}) = A_3 - \frac{S_4 \times A_2}{S_2}$$

$$\text{sodium estrone sulfate mg./Gm.} = \frac{A_{3\,(\text{corr.})} \times C_1 \times 1.38}{S_3 \times w}$$

where A_3 = absorbance of sample solution at 520 mμ, S_3 = absorbance of estrone standard solution at 520 mμ, S_4 = absorbance of equilin standard solu-

[1] Larger aliquots of solution B may be used for samples low in equilin. Do not exceed 25–30 mcg. of equilin.

tion at 520 mμ, C_1 = mg. estrone used in preparing the standard, and w = wt. of sample in Gm.

Paper Chromatographic Test for Identity.—Apparatus and reagents. Tank, U. S. P. XVI, p. 923, type 2. Chromatographic paper—8 × 8 in. sheets Whatman No. 1. Ultraviolet lamp—Chromato-Vue model 3-C, long wavelength, Black Light Eastern Corp., Bayside, N. Y. Immobile solvent—30 ml. of dimethylformamide (b. p. 152–154°), 20 ml. of formamide (stabilized-Fisher), and 50 ml. of methanol (anhydrous). Mobile solvent—40 ml. of methylcyclohexane (spectrophotometric grade), and 10 ml. of chloroform. Spray solution—(*a*) dissolve 20 Gm. of dry zinc chloride in 30 ml. of glacial acetic acid, (*b*) dissolve 1.00 Gm. of ferric chloride in 100 ml. of glacial acetic acid; mix 20 ml. of (*a*) and 3 ml. of (*b*) just prior to use. Standard solutions—Prepare methanol solutions of estrone, equilin, equilenin, estradiol 17(α), and estradiol 17 (β) containing 1.0 mg./ml.

Preparation of Sample Solution.—Evaporate 40 ml. of the benzene solution (*A*) to dryness on a steam bath with the aid of a current of air. Dissolve the residue in 2–3 ml. of chloroform and transfer with the minimum amount of chloroform to a 10-ml. glass-stoppered Erlenmeyer flask. Evaporate to dryness on a steam bath. Dissolve the residue in a quantity of methanol (based on the total estrogen assay) to produce a concentration of 1.0 mg. estrogens per ml.

Procedure.—Impregnate the paper by dipping in the immobile solvent, drain briefly, and blot between filter papers. After four minutes apply, with a micro pipet, 5-μL. spots of sample and standard solutions along a line one inch from bottom of the paper. Space the spots about one-half inch apart and not less than two inches from either side of the paper. While applying spots, evaporate the solvent with a stream of air. Instead of separate spots for each estrogen standard, a mixture of all may be applied as a single spot.[1]

Place the mobile solvent in a tray in the tank and insert the paper through the slot in the lid until it dips into the mobile solvent. One-half inch of paper should project above the lid. The paper can be secured by stainless steel pins. Allow the chromatogram to develop for two and one-half hours. If the relative humidity is less than 50%, place 5 ml. of water in a second tray, cover tank, and equilibrate for thirty minutes before starting chromatogram. At the completion of the development time, remove the paper from the slot and allow it to dry in air at least one hour.

Spray the paper thoroughly with spraying solution so that it is moist, but not dripping wet, and dry at 95–100° for five minutes. View immediately under ultraviolet light and circle the spots with a pencil. The compounds will separate and fluoresce as shown in Fig. 2. Conjugated estrogens from pregnant mares' urine may contain all of the above estrogens with estrone predominating.

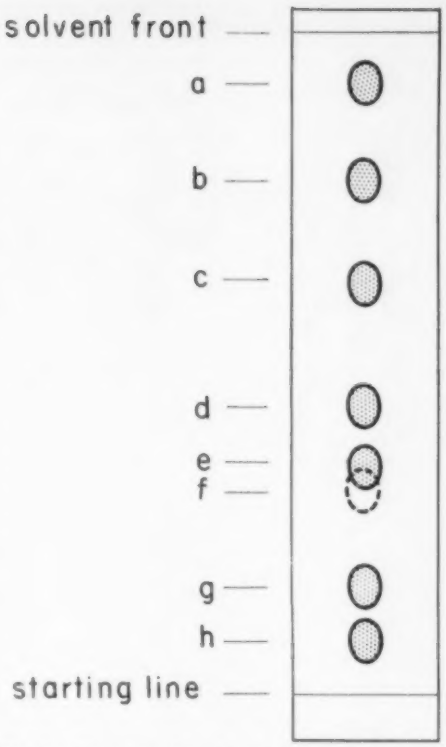

Fig. 2.—The separation of estrogenic steroids by the proposed paper chromatographic test.

	Compound	Fluorescence
a	estrone	blue
b	equilin	yellow-green
c	equilenin	rose
d	estradiol 17 α	orange
e	estradiol 17 β	blue-green
f	dihydroequilin 17 α	yellow
g	dihydroequilenin 17 α	yellow
h	dihydroequilenin 17 β	yellow

DISCUSSION

A series of alcoholic solutions was prepared containing estrone, equilin, and equilenin in the proportions generally found in commercial preparations. One-milliliter aliquots of these solutions were analyzed by the procedure described for sodium estrone sulfate and sodium equilin sulfate. The results (Table I) show excellent recoveries for estrone and equilin with little or no interference from equilenin.

A number of commercial conjugated estrogen tablets and powders were analyzed by the proposed

TABLE I.—DETERMINATION OF ESTRONE AND EQUILIN IN KETOSTEROID MIXTURES IN ALCOHOL

	—Estrone, mcg.—		—Equilin, mcg.—		Equilenin, mcg.
Sample	Added	Found	Added	Found	Added
1	52.1	52.5	..	0.6	5.0
2	44.0	44.5	4.0	4.2	2.5
3	38.7	37.9	2.3	2.5	7.6
4	29.2	29.5	13.8	13.7	5.2
5	23.2	23.9	26.7	25.9	2.6

[1] A convenient mixture of the standard solutions can be made using 1 ml. each of the estrone, equilin, and equilenin, 0.2 ml. estradiol 17α, 0.5 ml. estradiol 17β solutions.

TABLE IIa.—ANALYSES OF COMMERCIAL CONJUGATED ESTROGEN PREPARATIONS

Sample	—Total Estrogens as Sodium— Estrone Sulfate		Sodium Estrone Sulfate, mg.	Sodium Equilin Sulfate, mg.	Sum of Sodium Estrone Sulfate and Sodium Equilin Sulfate, % of Total
	Found, mg.	Declared, %			
Tablets					
0.3 mg.	0.330	110	0.208	0.091	90.6
0.625 mg.	0.682	110	0.418	0.166	85.6
1.25 mg.	1.32	105	0.741	0.364	83.7
1.25 mg.	1.34	107	0.840	0.300	85.0
1.25 mg.	1.35	108	0.805	0.341	84.9
1.25 mg.	1.40	111	0.753	0.479	88.0
1.25 mg.	1.39	110	0.738	0.427	83.8
1.25 mg.	1.31	104	0.701	0.372	81.9
1.25 mg.	1.28	101	0.909	0.207	87.2
1.25 mg.	1.20	96.0	0.965	0.110	89.6
1.25 mg.	1.19	95.2	0.960	0.093	88.5
Powder					
34.5 mg./Gm.	36.3	105	28.9	4.00	90.6
17.5 mg./Gm.	16.6	94.4	15.2	0.07	92.0
34.5 mg./Gm.	35.5	103	30.6	3.00	94.5
20 mg./vial	24.0	110	14.6	6.50	87.9

TABLE IIb.—REPRODUCIBILITY OF ANALYSES OF COMMERCIAL CONJUGATED ESTROGEN PREPARATIONS

Analyst	Sample A			Sample B		
	Total Estrogens as Sodium Estrone Sulfate, mg./Gm.	Sodium Equilin Sulfate, mg./Gm.	Sodium Estrone Sulfate, mg./Gm.	Total Estrogens as Estrone Sulfate, mg./Gm.	Sodium Equilin Sulfate, mg./Gm.	Sodium Estrone Sulfate, mg./Gm.
	36.4	3.6	30.0	5.04	1.69	2.64
1	36.0	3.8	30.0	5.03	1.70	2.70
	36.0	4.1	30.6	5.11	1.75	2.80
2	36.5	4.1	30.5	5.08	1.73	2.77
	36.2	3.9	30.4	5.16	1.78	2.66
3	36.2	3.9	30.7	5.16	1.77	2.69
Av.	36.2	3.9	30.4	5.10	1.74	2.71

method. The total estrogens, corrected for equilin, (Table IIa) show a reasonable compliance with the label declaration for each sample. The estrone plus equilin content was in all cases over 80% of the total estrogens and averaged 87.5%.

To obtain data as to the reproducibility of the procedure in the analysis of commercial estrogenic preparations, two samples were assayed in duplicate by three analysts. Sample A powder labeled to contain 34.5 mg. of conjugated estrogens per Gm. Sample B was prepared from coated tablets, powdered to 60 mesh, and thoroughly mixed. Based on the average weight determination these tablets should contain 5.0 mg. per Gm. These results (Table IIb) show excellent checks for each analyst, and very good overall agreement between analysts.

The proposed method was found suitable for the assay of pharmaceuticals containing conjugated estrogen in combination with other active drugs, or in dosage forms other than tablets. In most of these preparations, the estrogens constituted a minor portion of the total drug content. In some instances, slight modifications of the proposed method were necessary. For the analysis of the syrup, a 10-ml. sample was incorporated in 10 Gm. of siliceous earth and larger aliquots were used in the colorimetric determinations. The assay of the conjugated estrogen-meprobamate combination utilized a sample equivalent to two tablets. Suitable alterations in the volume of the solutions and aliquots were made to obtain satisfactory levels of intensities in the colorimetric steps. In these assays, phenobarbital, meprobamate, methyltestosterone, and other chloroform-soluble drugs, are removed in the initial 100-ml. chloroform wash of the column. The results of these analyses (Table III) are in good agreement with the labeled declaration.

A comparison of analyses for estrone and equilin in commercial preparations by the proposed method and by the A.O.A.C. procedure (2) is presented in Table IV. The data show a good correlation between the two methods.

Qualitative paper chromatographic identity tests were applied to extracts from many conjugated estrogen preparations and excellent separation of the individual steroids was obtained (Fig. 2). The spots fluoresced brilliantly and were colored as indicated. Examination must be made immediately after drying since the fluorescence diminishes rapidly. As little as 0.1 mcg. of the diols and 0.5 mcg. of the ketosteroids can be detected in the individual spots.

SUMMARY

1. A method has been proposed for the determination of total estrogens, estrone, and equilin in conjugated estrogen preparations.

2. The conjugates are extracted and sepa-

TABLE III.—ANALYSIS OF COMMERCIAL PHARMACEUTICALS CONTAINING CONJUGATED ESTROGENS IN MIXTURES

Sample	Total Estrogens as Sodium Estrone Sulfate, mg.	Sodium Estrone Sulfate, mg.	Sodium Equilin Sulfate, mg.
Syrup			
Conjugated estrogens, 0.625 mg./4 ml.	0.744	0.382	0.195
Tablets			
Conjugated estrogens, 0.625 mg. Methyltestosterone, 10 mg.	0.699	0.385	0.208
Conjugated estrogens, 1.25 mg. Phenobarbital, 32 mg.	1.32	0.693	0.429
Conjugated estrogens, 1.25 mg. Methyltestosterone, 10 mg. Ascorbic acid, 400 mg.	1.29	0.690	0.440
Conjugated estrogens 0.4 mg. Meprobamate, 400 mg.	0.423	0.253	0.129

TABLE IV.—COMPARISON OF PROPOSED PROCEDURE AND A.O.A.C. METHODS FOR THE DETERMINATION OF ESTROGENIC KETOSTEROIDS

Sample	Estrone		Equilin	
	Proposed	A.O.A.C.	Proposed	A.O.A.C.
Conjugated estrogen tablets, 1.25 mg. as sodium estrone sulfate	0.620 mg./tab.	0.650 mg./tab.	0.228 mg./tab.	0.203 mg./tab.
	0.586 mg./tab.	0.570 mg./tab.	0.244 mg./tab.	0.245 mg./tab.
	0.548 mg./tab.	0.534 mg./tab.	0.338 mg./tab.	0.335 mg./tab.
Conjugated estrogen powder, 12.0 mg. estrone equivalent per Gm.	10.95 mg./Gm.	10.45 mg./Gm.	0.05 mg./Gm.	0.05 mg./Gm.

rated using a simple siliceous earth column. Colorimetric determinations are made with new modifications of the Kober reaction.

3. The new procedure is simpler, faster, and more sensitive than previous methods.

4. A paper chromatographic method is described that separates all of the estrogens that normally occur in mixtures from pregnant mares' urine.

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Effect of Gonadectomy and Estradiol Benzoate Administration on the Minimal Carcinogenic $Dose_{50}$ of Methylcholanthrene on Mouse Epidermis

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The results indicate that gonadectomy without further treatment, other than the application of the MCD_{50} of methylcholanthrene, did not affect the tumor incidence. Intact or gonadectomized females showed an increased tumor incidence when subjected to the MCD_{50} of methylcholanthrene and biweekly injections of 1 mcg. of estradiol benzoate in sesame oil, in contrast to intact or gonadectomized males, which, under similar conditions, showed a decreased tumor incidence. The variation in the tumor incidence of intact and gonadectomized male and female mice under estrogen treatment was such that the total tumor incidence still approximated 50 per cent.

ALTHOUGH THE EFFECTS of certain hormones, such as cortisone (1) and hydrocortisone (2), on the occurrence of chemically induced epidermal tumors in mice have been clearly elucidated, a paucity of fundamental information still exists in the literature concerning the influence exerted by estradiol and other gonadal hormones on the incidence of epidermal carcinomas in laboratory animals. On the other hand, voluminous data are available with respect to estrogenic involvement in the influence and/or production of many other types of malignant transformations or growths (3).

Both injected estrogen (4) and topically-applied methylcholanthrene (5) have been shown to increase markedly the mitotic rate of the anterior dorsal epidermis of mice. Because estrogen is involved in both carcinogenesis and anticarcinogenesis, it becomes imperative to ascertain the part played by estrogen when it is administered to both sexes in conjunction with methylcholanthrene in a satisfactory dose/response relationship.

It has been previously established and confirmed in this laboratory (6) that the minimal carcinogenic $dose_{50}$ (MCD_{50}) of methylcholanthrene to induce squamous cell carcinomas on the epidermis of CF-1 mice is 504 mcg. applied through twenty-one biweekly applications of 0.02 ml. of a 0.12% solution of methylcholanthrene in acetone. Obviously, the next step would be the use of the standardized dose/response technique as a basis for determining inhibition or enhancement of tumor growth in different experimental procedures. Therefore, it is the purpose of this investigation to observe the effect of (*a*) gonadectomy, (*b*) estrogen administration, (*c*) both gonadectomy and estrogen administration concurrently on the previously established tumor incidence in response to the minimal carcinogenic $dose_{50}$, and (*d*) to reconfirm this dose (the MCD_{50}) in control animals.

MATERIALS AND METHODS

The experimental plan required a repetition of the procedures (part I and part II) in order to verify the consistency and reliability of the tumor incidence in response to the MCD_{50}.

In each part, 120 CF-1 albino mice (60 males and 60 females), approximately ten weeks old, were divided into four groups of 30 mice, each with 15 males and 15 females. Consequently, there were 240 mice used in the entire study with each group consisting of a total of 60 animals, equally divided as to sex. The cages employed, diet, handling, shaving, weighing, and method of application of the carcinogen are described in a previous paper (6).

Five hundred milliliters of a 0.12% solution of methylcholanthrene in acetone were prepared. In order to facilitate the handling of the methylcholanthrene solution, 4-ml. ampuls were made and stored in a refrigerator at 0–5° until used.

In order to maintain an elevated estrogen level, which increases epidermal mitotic activity, it was decided to inject lower physiological doses of estrogen rather than extremely high doses, to avoid the possibility of toxic reactions that might arise from the chronic administration of larger amounts (7). After consulting the findings of Liu (8), who observed that estrogenic activity did not increase in rats or mice injected with doses greater than 0.6 γ of estradiol, 1 mcg. was regarded as a low standard dose of estrogen for this experiment. It was presumed that this dose of estrogen would not cause the toxic responses reported by Gardner (7), yet still be high enough to exert effects that are characteristic of elevated estrogen levels (i.e., estrus), which could easily be ascertained at any time in the experiment by the vaginal smear technique.

In preliminary experiments a solution of estradiol benzoate in sesame oil containing 10 mcg. in each

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milliliter was prepared. One-tenth of a milliliter of this solution (containing 1 mcg. of estradiol) was injected into the inguinal subcutaneous tissue of female mice. The absorption of the estrogen was proved by the appearance of keratinized epithelial cells, which are indicative of estrus, in vaginal smears derived from fluid aspirated from the injected animals. It was noted that the mice remained in a state of estrus for three days and nineteen hours with one injection of the above quantity of estrogen before resuming their regular cycle. Since it was desired to keep the mice in a state of continuous estrus, additional mice were subjected to estrogenic treatment approximately every three and one-half days. These animals remained in a constant state of estrus under this regimen. Therefore, it was concluded that this quantity of estrogen and frequency of administration would satisfy the requirements of the animals in the experimental groups designated to receive the female sex hormone.

Gonadectomies were performed by common laboratory procedures by means of a semisterile technique and ether anesthesia. After a ten-day period of recovery, these animals were made ready for the main experiment.

EXPERIMENTAL

In parts I and II of this investigation there were four groups of 30 mice labeled A, B, C, and D, respectively. Group A contained the control animals, which were used to reconfirm the MCD_{50} previously reported in this laboratory. The animals in group B were gonadectomized and subjected to only the MCD_{50} of methylcholanthrene. The mice in group C were intact and received not only the MCD_{50} of methylcholanthrene, but also injections of estradiol benzoate to maintain high estrogen levels. The animals in group D were gonadectomized and received the MCD_{50} as well as injections of estradiol.

All of the mice in both phases of the experiment received 0.02 ml. of a 0.12% solution of methylcholanthrene in acetone twice weekly (Tuesdays and Thursdays) applied to the shaved interscapular region. Each mouse that received 1 mcg. of estradiol benzoate in 0.1 ml. of sesame oil twice weekly (Mondays and Fridays) was injected in subcutaneous tissue, alternating the site among the folds of the four limbs.

With the appearance of the first growth, all of the mice were carefully examined four times a week with a model 701-A Stanley magnifying glass. At the end of both phases of the experiment, the tumors were drawn and recorded on special data sheets which gave all the pertinent information for each mouse.

During the entire experiment, four hundred vaginal smears were prepared according to the method of Allen (9), in order to observe (*a*) normal cyclical activity in the control groups, (*b*) the phase of continuous diestrus in the gonadectomized groups receiving the MCD_{50} alone, and (*c*) the state of continuous estrus in the groups receiving estradiol benzoate injections.

At the termination of each phase of the experiment, several mice were selected from the respective groups that showed evidence of typical tumorous growths. These mice were chloroformed and the tumors, mammary glands, and uteri from each mouse were excised and immersed in 10% formalin solution in preparation for eventual sectioning and microscopic pathological examination.

All essential data were assembled and arranged into four tables. Only those tumors were recorded that measured 1 mm. or more in at least one dimension (width *versus* height). Growths that did not conform to this criterion were extremely difficult to identify as squamous cell carcinomas. Table I (part I) and Table II (part II) show the composite results with respect to the separate groups at the termination of the experimental phases. Table III shows the initial and termination weight and standard deviation for the animals in the separate groups (part I and part II). Table IV shows the total results obtained by combining the entire data of part I with that of part II.

Epilation was evident in all intact animals beginning about five to six days after the first application of the carcinogen. The epilation progressed for nineteen days, at which time many animals showed

TABLE I.—COMPOSITE RESULTS OF THE DIFFERENT GROUPS

Group	Total Methylcholanthrene Administered, mcg.[a]	Effective Total (Mice/Group)	Tumorous Mice per Group	Tumor Incidence, %	Sex	
					Male	Female
A	336–504	27	15	55.5	8	7
B	312–504	29	16	55.2	7	9
C	336–504	30	16	53.3	7	9
D	336–504	30	17	56.6	7	10

[a] The first number is the smallest quantity, in micrograms, of methylcholanthrene necessary to induce a tumor in at least one animal in that group. The second number is the total amount, in micrograms, of methylcholanthrene applied to all animals at the completion of the experiment.

TABLE II.—COMPOSITE RESULTS OF THE DIFFERENT GROUPS

Group	Total Methylcholanthrene Administered, mcg.[a]	Effective Total (Mice/Group)	Tumorous Mice per Group	Tumor Incidence, %	Sex	
					Male	Female
A	288–504	30	16	53.3	9	7
B	288–504	30	15	50.0	9	6
C	288–504	29	15	51.7	6	9
D	384–504	30	12	40.0	4	8

[a] The first number is the smallest quantity, in micrograms, of methylcholanthrene necessary to induce a tumor in at least one animal in that group. The second number is the total amount, in micrograms, of methylcholanthrene applied to all animals at the completion of the experiment.

practically complete epilation of the interscapular region as well as of the surrounding area, to which the carcinogen spread by means of capillarity. For the next few days, this nearly complete epilation persisted and was then followed by a period of slight stimulation of hair growth that continued for one week, whereupon these mice underwent a resurgence of hair growth that left them with longer hair in the interscapular region than in other areas of their bodies, even though this hair was clipped once each week.

The hair growth pattern followed a different course in the gonadectomized animals. They all responded to the depilatory effects of methylcholanthrene for the first ten days. At this time these animals began to exhibit a stimulation of hair growth which was opposite to the marked epilation observed in the intact mice at the same phase of the experiment. This resurgence of hair growth only lasted for seven days at which time profound epilation commenced. From the nineteenth day on, the hair growth pattern resembled very closely that found in the intact animals.

Seven days after the first application of the carcinogen, a slight vasodilation of the subcutaneous blood vessels was observed with the naked eye. This vasodilation was noted in all animals irrespective of the group and continued throughout the duration of the experiment. In certain animals, subcutaneous hemorrhages occurred which occasionally broke the surface of the epidermis, leaving a weeping lesion.

Vaginal smears prepared from the different groups confirmed the fact that three groups were maintained at a particular phase of the estrus cycle while the control group was shown to react cyclically in a normal manner, as anticipated. Those mice that were ovariectomized and received no estrogen (group B) remained in a relatively stable diestrus state, i.e., vaginal smears from this group showed few epithelial cells, many leukocytes, and much mucus, all of which are indicative of diestrus. In several cases, after fifty-six days had elapsed in the experiment, some of the ovariectomized mice in this group exhibited a return to normal cyclic activity. The typical smear at this time revealed a confused picture with no definite phase of the estrus cycle clearly distinguishable. There appeared to be an intermingling or an overlapping of the phases into one that was indistinct. However, this confusion was transient and was followed by a return in a few days to a continuous diestrus state. A recurrence of this phenomenon was evident several times during the latter stages of this investigation in the animals of this group.

Those intact mice that were injected with estrogen remained in a constant state of estrus, i.e., vaginal smears prepared from these animals showed almost exclusively keratinized squamous epithelial cells, which are indicative of estrus. Similarly, ovariectomized animals subjected to estrogen injection also remained at a relatively stable estrus phase.

Of the 240 animals employed in both phases, three males and two females died. It was not possible to ascertain the cause of death in these animals. All deaths seemed to be due to factors unrelated to the experiment. The animals that died showed no signs of injured epidermis or hemorrhage in the interscapular region, which was the area immediately exposed to the action of the carcinogen. The mice that succumbed showed neither mild nor severe pathological symptoms right up to the time of death. In fact, eight hours prior to death they were regarded as normal in all respects.

Pathological examination of tumors, uteri, and mammary glands from several tumorous mice in the respective groups was accomplished by making H and E sections of these tissues. The tumors that were induced were true cancers, typical of those produced by methylcholanthrene on epidermis, *viz.*, squamous cell carcinomas. None of the mammary glands or uteri from the mice in groups A and B showed any evidence of abnormal change. Also, none of the mammary glands or uteri from the mice in groups C and D showed any evidence of possible abnormal changes known to occur following prolonged estrogenic stimulation.

Table III.—Initial Weight Compared with Termination Weight

Group 30 Mice/Group	Part I Mean Weight, Gm.	Part I Standard Deviation	Part II Mean Weight, Gm.	Part II Standard Deviation
A[a]	25.4	±3.5	26.4	±4.0
A[b]	30.7	±4.1	29.7	±4.5
B[a]	26.5	±3.3	27.1	±2.0
B[b]	32.5	±2.5	31.3	±2.5
C[a]	25.1	±2.8	26.4	±3.2
C[b]	31.1	±2.3	30.4	±3.0
D[a]	26.0	±2.5	28.7	±2.9
D[b]	30.7	±3.2	30.2	±3.1

[a] Initial weight. [b] Termination weight.

DISCUSSION

The results in both phases of the experiment, as shown in Tables I and II, were similar in group A. These animals were used to reconfirm the MCD_{50} of methylcholanthrene that had previously been reported (6), and also served as controls for this investigation. The total tumor incidence obtained by combining group A of part I and II was 54.4%. The effective total of 57 animals in group A (Table IV) showed a tumor distribution, according to sex, of 17

Table IV.—Total Results for Part I and Part II

Group	Total Methylcholanthrene Administered, mcg.[a]	Effective Total (Mice/Group)	Tumorous Mice per Group	Tumor Incidence, %	Sex Male	Sex Female
A	288–504	57	31	54.4	17	14
B	288–504	59	31	52.5	16	15
C	288–504	59	31	52.5	13	18
D	336–504	60	29	48.3	11	18

[a] The first number is the smallest quantity, in micrograms, of methylcholanthrene necessary to induce a tumor in at least one animal in that group. The second number is the total amount, in micrograms, of methylcholanthrene applied to all animals at the completion of the experiment.

males and 14 females. Thus, the percentage of tumor incidence agreed with the previous findings in this laboratory (6), although the response according to sex did not conform with the results of the earlier study in which twice as many female mice developed tumors as males.

As a result of the previous investigation it was hypothesized that females were probably more susceptible to this dose of methylcholanthrene than males due to increased mitotic activity in their anterior dorsal epidermis which occurs during the estrus cycle. Therefore, it was believed that the larger number of female mice with tumors could be attributed directly to the effect of the mitogenic hormone, estradiol, which stimulates cellular division and could conceivably raise the tumor incidence. However, in this study, this does not appear to be the case for, when past and present data are compared, the only similarity that is apparent is the overall tumor incidence occurring among CF-1 mice of mixed sex treated with the MCD_{50} of methylcholanthrene, which indicates that sex did not exert a primary influence on the final response. It is therefore assumed that the tumor incidence would have remained constant even if animals all of one sex had been used.

The data presented in Table IV indicate that gonadectomy without further treatment other than the application of the MCD_{50} of methylcholanthrene did not affect the tumor incidence, for 52.5% of the animals in group B showed tumor involvement. This corresponds to the 54.4% observed in the intact animals (group A). Also, in group B the distribution of the tumor incidence did not favor either sex. Therefore, it may be concluded that any decreased epidermal mitotic activity caused by ovariectomy did not retard the chemical induction of tumors in this group. It may also be concluded that castration did not alter the tumor incidence in male mice since there were 15 males with tumors in group B and 14 in group A.

As revealed in Table IV, the tumor incidence in groups C and D was higher for female mice. This may be regarded as significant because the tendency toward a higher tumor incidence in the female mice of these groups occurred in both part I and II, as shown in Tables I and II. Since estrogens are known to be highly carcinogenic (3), it appears that the greater tumor incidence among females in both these groups (C and D) is due to the biweekly injection of the female sex hormone which was administered to these groups. The fact that the total tumor incidence decreased among males in groups C and D can be attributed to an actual inhibition of chemically-induced tumors by estrogen in low physiological dosage. These two assumptions are based upon the well established evidence that estrogens are involved in carcinogenesis and anticarcinogenesis (10).

The dose of estrogen administered in this study evidently was not carcinogenic to the male animals because their tissues were insensitive to the mitogenic effects of this hormone. However, if larger doses of estrogen had been used, the tumor incidence would undoubtedly have increased (11, 12). Conversely, the tumor incidence was higher in female mice perhaps because their tissues were more sensitive to the carcinogenic and mitogenic effects of the injected estrogen. Although more females responded with tumors when treated with estrogen, the inhibition of carcinogenesis in males under the same treatment offset the increase in females, so that the combined tumor incidence of each respective group approximated that observed with the MCD_{50} in groups A and B (Table IV).

From Tables III and IV it is obvious that the procedures outlined herein were not deleterious to the general health of the animals for, not only did practically all of the animals survive, but they also gained a relatively equal amount of weight. The increase in weight and the number of surviving animals is evidence that the mice did not at any time exhibit the incapacitating effects associated with the chronic administration of estrogen (7). Consequently, the dose of estrogen used in this experiment accomplished what was expected of it, without causing abnormalities that would have confused the evaluation of the data.

The hair growth pattern among intact mice was very similar to that which was reported in this laboratory (6). However, the hair growth pattern varied in the early stages of the experiment for the gonadectomized animals of either sex. They all exhibited, after an initial ten-day period of epilation caused by methylcholanthrene, a stimulation of hair growth for one week. This response was in direct contrast to that which occurred in intact mice, leading one to hypothesize that something responsible for the steady loss of hair for the first nineteen days with methylcholanthrene is lost with gonadectomy. This effect was transitory, however, for, after the one-week period of hair growth stimulation, they reverted to the depilatory stage for a short time and then underwent a resurgence of hair growth, as did the intact mice, for the remainder of the experiment.

The occurrence of a return to a normal cycle in spayed mice is recorded in the literature. Perry (13) and Wolley, *et al.* (14), have reported this phenomenon. The latter authors' findings suggest that adrenal cortical estrogen is the necessary stimulus for this return to a partially normal cycle in ovariectomized mice after a prolonged diestrus interval. A pseudoestrus state can occur in spayed mice, which undoubtedly was the case in this study as depicted by vaginal smears from a few ovariectomized animals that occasionally yielded confused and indistinct phases.

Mice that received injections of the female sex hormone showed neither toxic effects, abnormal growths in the mammary glands, nor malignant transformations in the uteri. Therefore, it may be concluded that the estradiol dosage employed in this experiment was innocuous to the recipient animals, although it caused a continuous estrus and stimulated the mitotic rate in the epidermis of female mice, which aided in the formation of more tumors in this sex.

SUMMARY

1. The previously established minimal carcinogenic $dose_{50}$ of methylcholanthrene necessary to induce squamous cell carcinomas on the epidermis of CF-1 mice, 504 mcg. applied through twenty-one biweekly applications of 0.02 ml. of a 0.12 per cent solution of methylcholanthrene in acetone, was reconfirmed in two separate studies.

2. This standard dose/response relationship can be employed as a basis for the determination of factors concerned with the enhancement or inhibition of carcinogenesis.

3. Gonadectomy alone did not modify the tumor incidence in response to the MCD_{50}.

4. Intact or gonadectomized female animals showed an increased tumor incidence when subjected to the MCD_{50} of methylcholanthrene and biweekly injections of 1 mcg. of estradiol benzoate in sesame oil, in contrast to intact or gonadectomized male animals, which under similar conditions showed a decreased tumor incidence.

5. The variation of the tumor incidence of intact and gonadectomized male and female mice under estrogen treatment was such that the total tumor incidence still approximated 50 per cent.

6. Biweekly injections of 1 mcg. of estradiol benzoate in sesame oil were sufficient to maintain estrus for at least ten weeks.

7. The transient return to a state of pseudo estrus in ovariectomized mice accounted for the appearance of keratinized epithelial cells in vaginal smears obtained from some mice approximately fifty-six days after ovariectomy.

8. The estrogen dosage employed in this experiment was apparently beyond the physiologic range, yet below the toxic level. This was substantiated by the large number of living mice in an apparently healthy state.

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Studies on Larch Arabogalactan I

By M. R. NAZARETH, C. E. KENNEDY, and V. N. BHATIA

Arabogalactan is a carbohydrate substance extracted from the stems of *Larix occidentalis*. Its properties and the properties of its aqueous solution have been studied, the LD_{50} of the compound has been determined, and a thirty-day feeding study has been carried out in which it formed 0, 2, 10, and 25 per cent of the diet of groups of albino rats. The results indicate that arabogalactan is essentially nontoxic. Its use as an emulsifier has been studied and shows it to be useful; particularly for the production of stable, low-viscosity emulsions.

LARCH ARABOGALACTAN is a water-soluble polysaccharide occurring as a natural gum in various species of the genus *Larix*, and is also found in small quantities in other conifera. Several workers (1–4) have shown that arabogalactan contains arabinose and galactose and that the proportion of sugars in the molecule is about one to six, respectively. Bouveng and Lindberg (5, 6) have elucidated some of the molecular structure through hydrolysis and methylation studies. The literature up to 1959 has been summarized by Stout (7). Western Larch, *Larix occidentalis*, is the best commercial source, containing from 8 to 25 per cent of arabogalactan on a dry wood basis (8).

Larch arabogalactan accumulates in masses under the bark of the living tree as the result of injuries and has a high degree of purity (95%). This source cannot compete in volume or in price with methods based on the extraction of the chipped wood (9–11). An efficient method for the commercial production of Larch arabogalactan has been recently developed by the Division of Industrial Research of Washington State University under the direction of M. F. Adams. The patent for this process is pending.

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Presented to the Scientific Section, A. PH. A., Washington, D. C., meeting, August 1960.
The authors wish to express their thanks to Dr. Mark F. Adams, Head of the Chemistry-Spectroscopy Sections of the Division, under whose supervision this work was done. Dr. Adams also supplied the data in Figs. 1 and 7.
The gross and histopathological examination of the animals used in the short term toxicity studies were carried out by Dr. Roger G. Spencer, Chairman of the Department of Veterinary Pathology, Washington State University.
Statistical evaluation of data for the LD_{50} determination was done by Dr. Thomas S. Russell, Associate Statistician, Washington Agricultural Experiment Stations, Washington State University.

EXPERIMENTAL

Properties

The spray dried product obtained from aqueous extracts is a fluffy, buff colored powder with an aromatic odor reminiscent of freshly cut wood. The purity of this substance is about 97%. Small experimental batches of a pure white sample of over 99% purity have been prepared, and its commercial preparation is under study (12). The data reported in this paper are based on studies with the 97% pure material. Arabogalactan has been reported as consisting of two fractions having molecular weights of 16,000 and 100,000 each (6). The sample used in this study had an average molecular weight of approximately 20,000, as determined by the Ostwald pipet method.

Solubility.—Arabogalactan is insoluble in oils and in most organic solvents. It is freely soluble in water, dissolving completely in hot or cold water, forming an amber solution with a pH of 4–4.5. Solutions containing up to 60% w/v of arabogalactan can be easily prepared and solubility increases with increasing temperature.

rabogalactan solutions (25%) can withstand as much as 70% of its own volume of absolute alcohol without precipitation if it is added slowly and with continuous stirring.

Type of Flow.—Arabogalactan solutions exhibit an apparent Newtonian flow over the sheer rate range studied when flow characteristics were determined using a Brookfield Synchro-Lectric viscosimeter model LVF. The results are shown in Fig. 1.

Viscosity.—The effect of concentration on the viscosity of arabogalactan solutions is shown in Fig. 2. The viscosities were measured with an Ostwald viscosimeter at 20 ± 0.5°. The efflux time was two hundred seconds or more. The data represents the results of repeated determinations to assure reproducibility. Arabogalactan differs from most gums in that it forms solutions of very low viscosity even in high concentrations.

Effect of Temperature.—The effect of temperature on the viscosity is shown in Fig. 3. An increase in temperature causes a lowering of viscosity.

Effect of Electrolytes.—Sodium iodide, sodium hydroxide, hydrochloric acid, sodium bicarbonate, sodium sulfate, and calcium chloride were added to a 5% w/v solution and a 20% w/v solution of arabogalactan at 20 ± 0.5°. The concentration of the salt was 0.1 *M*. These electrolytes had no significant effect on the viscosity of arabogalactan as determined using an Ostwald viscosimeter.

Effect of Aging.—Aging has no deleterious effect on the viscosity of unpreserved arabogalactan solutions. A 40% w/w unpreserved solution of arabogalactan showed no loss in viscosity when checked over a period of one month at room temperature.

Effect of pH.—The viscosity of a 5% w/v and a 20% w/v solution of arabogalactan at a pH range

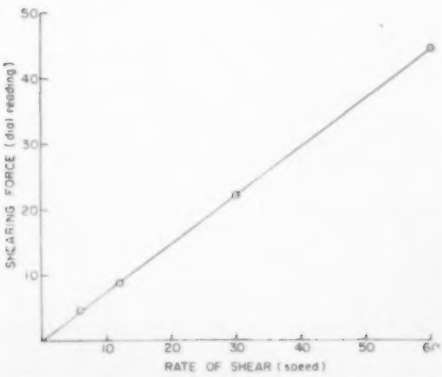

Fig. 1.—Rate of shear vs. shearing force on a 40% w/w arabogalactan solution at 25 ± 0.5° (using a Synchro-Lectric viscosimeter, No. 1 spindle).

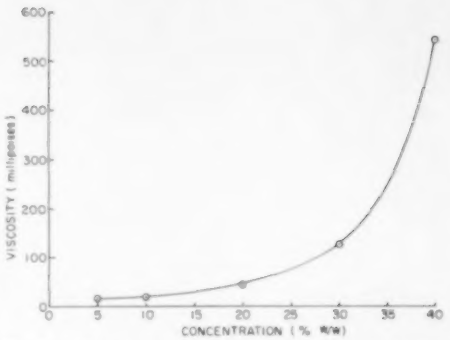

Fig. 2.—Viscosity vs. concentration of arabogalactan at 20° ± 0.5°.

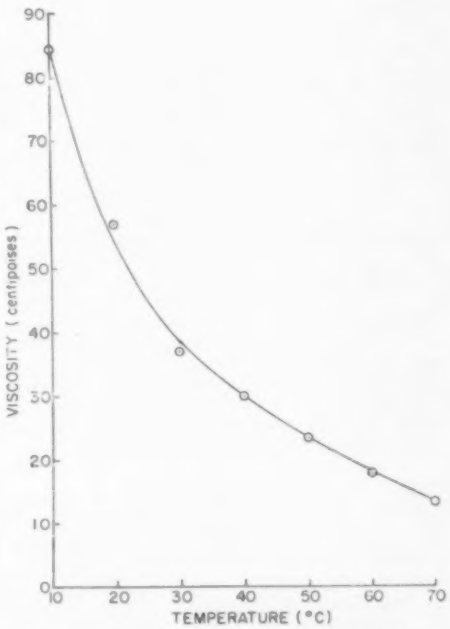

Fig. 3.—Viscosity of 40% w/w arabogalactan as a function of temperature (using a Brookfield Synchro-Lectric viscosimeter).

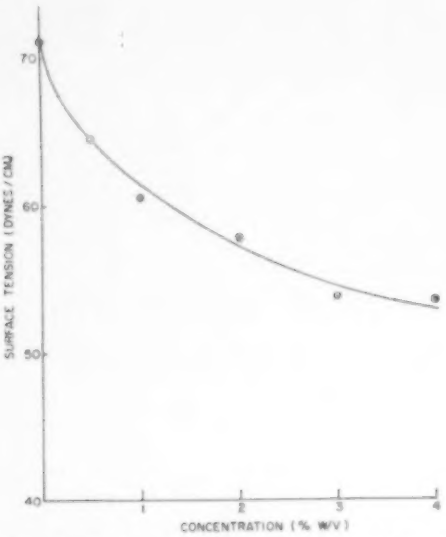

Fig. 4.—Surface tension vs. concentration of arabogalactan at 20 ± 0.5°.

from 1.5 to 10.5 was determined at 20 ± 0.5° using an Ostwald viscosimeter. pH does not significantly affect the viscosity of arabogalactan solutions. The pH was adjusted with sodium hydroxide and hydrochloric acid.

Surface Tension.—The addition of arabogalactan to water results in the lowering of surface tension, as shown in Fig. 4.

Effect of Electrolytes.—The addition of electrolytes lowers the surface tension with sodium hydroxide exerting the greatest depressing effect, as shown in Table I.

Table I.—Effect of Electrolytes on Surface Tension of 0.5% w/v Arabogalactan at 20 ± 0.5°[a]

Electrolyte (0.5 *M*)	Surface Tension, dynes/cm.
No electrolyte	63.6
Sodium hydroxide	51.0
Sodium iodide	57.1
Sodium bicarbonate	57.2
Sodium sulfate	58.7
Hydrochloric acid	61.5

[a] Determination made using a DuNoüy tensiometer.

Interfacial Tension.—The interfacial tension between liquid petrolatum and various arabogalactan solutions was determined. The results are shown in Fig. 5. As the concentration of arabogalactan is increased there is a definite decrease in interfacial tension which tends to level off at a concentration of 4% w/v.

Freezing Point.—The freezing point depression produced by arabogalactan in solution is shown in Fig. 6 and the data represents the results of repeated determinations to assure reproducibility.

Refractive Index.—The refractive index of various arabogalactan solutions has been determined using

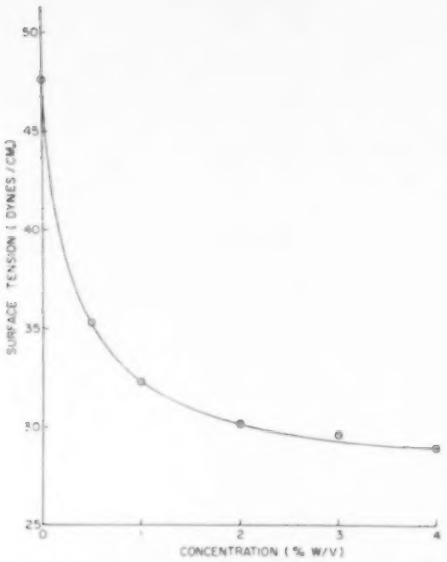

Fig. 5.—Interfacial tension between arabogalactan solution and heavy liquid petrolatum vs. concentration at 20 ± 0.5° (using a DuNoüy tensiometer).

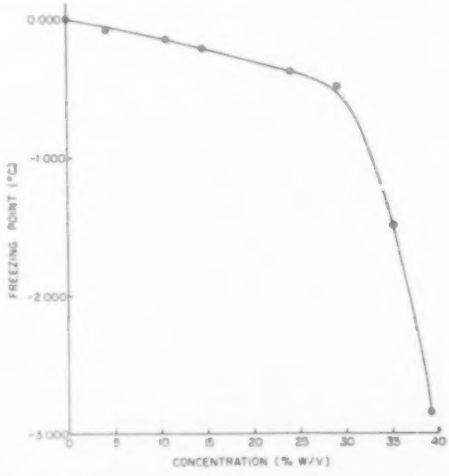

Fig. 6.—Freezing point of arabogalactan solutions vs. concentration (using Beckman thermometer).

an Abbe refractometer at 20 ± 0.5°. The linearity of the refractive index as a function (Fig. 7) of concentration is used as an analytical tool in the extraction process.

Toxicity

Acute Toxicity.—The acute toxicity of arabogalactan in rats is very low. In a series of experiments on 108 adult male albino rats in which arabogalactan was administered in aqueous solution intraperitoneally, the LD_{50} proved to be of the order of 12.9 Gm./Kg. Statistical treatment of the data

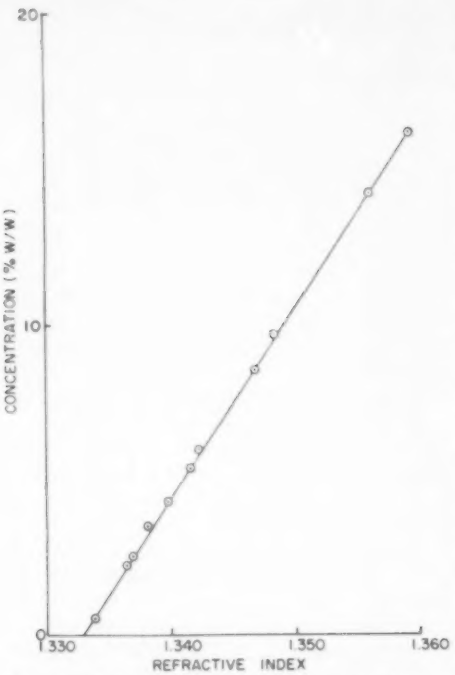

Fig. 7.—Refractive index vs. concentration of arabogalactan at 20 ± 0.5°.

according to the method of Litchfield and Wilcoxon (13) gave doses in Gm./Kg with 19/20 confidence limits as follows: LD_{16}, 10.8; LD_{50}, 12.9; LD_{84}, 15.4.

Preliminary Chronic Toxicity.—Groups of 10 male and 10 female weanling (age twenty-eight days) rats were fed diets containing 0, 2, 10, or 25% arabogalactan for thirty days. A slight diarrhea was observed in the 25% group, but there was no evidence found of interference with growth, which remained relatively uniform for all groups. Pooled samples of urine were examined weekly for sugar, protein, and blood, and were found to be normal. At the end of the thirty-day feeding period four male and four female rats were selected at random from each group and sacrificed by pithing. A complete necropsy was performed immediately. There were no gross lesions seen of any significance. Pieces of heart, lung, liver, kidney, intestine, stomach, brain, larynx, and occasionally other organs, were fixed in 10% formalin for histopathologic examination. On examination, these tissues revealed no significant lesions associated with the feeding of the material. A few enlarged foci of lymphocytes were found around the bronchi but these lesions were seen in both controls and test animals and are not considered related to the feeding. A complete blood examination was also performed immediately after sacrifice of the animals. Blood examinations gave variable results in all groups including the control but are probably normal for the age and strain of rats. Some of the variations are of the type that might result from contamination of the blood by lymph, e. g., variations in duplicate hematocrit readings.

The preliminary short term studies indicate that arabogalactan is innocuous to rats when fed for thirty days up to 25% of the ration.

Emulsifying Action

Arabogalactan is a good emulsifier, forming stable, low-viscosity emulsions. A series of liquid petrolatum emulsions were made with arabogalactan and with acacia using the following proportion of oil, water, and gum for the primary emulsions: 4:2:1, 6:3:1, and 8:4:1. The primary emulsions were made in a Waring Blendor and then diluted to contain equal parts of water and oil. The emulsions made with arabogalactan were less viscous compared to the ones made with acacia, e. g., the final emulsions using a 4:2:1 ratio of oil, water, and gum (in the primary emulsion) had a viscosity of 46 cps. with arabogalactan (Brookfield Synchro-lectric viscosimeter, spindle No. 1, 12 r. p. m.) as compared to 600 cps. (using No. 3 spindle) for the similar acacia emulsion.

An eight-week stability study on the globule size at 4°, room temperature, and 50° showed no difference between the two sets of emulsions made with arabogalactan and acacia. As expected, there was a slight increase in globule size at 4° and 50° in both sets of emulsion, the initial values being 4μ in all the emulsions, while the final values at 4° and 50° were 5μ while there was no noticeable change in the emulsion kept at room temperature. Arabogalactan is therefore as capable of making stable emulsions as acacia, but may be of much more value where emulsions of low viscosity are desired.

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Studies on Larch Arabogalactan II

By M. R. NAZARETH, V. L. NARAYANAN, and V. N. BHATIA

Some of the uses of arabogalactan in tableting have been studied. Its action as a binding agent appears to be satisfactory. The stability of a number of tablets made with arabogalactan as the binder have been compared with similar tablets made with acacia. The results are encouraging. Arabogalactan has been partially methylated to give methyl arabogalactan. Arabogalactan acetate has been made by a new procedure.

LARCH ARABOGALACTAN is a natural gum found in various species of the genus *Larix*, of which Western Larch, *Larix occidentalis*, is the best commercial source. Its physical properties, toxicity, and emulsifying action have been reported (1).

The adhesive character of Larch arabogalactan suggested its use as a tablet binder. Investigation of this aspect is reported here and it appears to be a good tablet binder. In order to make sure that when used as a binder it did not have any deleterious effects on the active ingredients, a number of tablets (vitamin B_2, folic acid, vitamin C, vitamin K, and aspirin) made with arabogalactan were compared with similar tablets made with acacia.

To determine the binding capacity of arabogalactan, soluble (lactose) and insoluble (lactose-calcium phosphate) tablets were made with arabogalactan as the binding agent. Similar tablets were made under identical conditions with acacia as the binder. The tablets made with arabogalactan and acacia were compressed to the same thickness and weight, and at the same pressure setting on the tablet machine (Stokes B-2 rotary). Under these conditions the hardness of the tablets served as a measure of the binding action.

Various chemical modifications of arabogalactan are being made and studied, the object being to make the substance more lipophilic and soluble in organic solvents. Similar work has been carried out with sucrose (2) resulting in the preparation of useful compounds. This paper includes a report on the preparation of arabogalactan acetate and a preliminary report on the partial methylation of arabogalactan.

Peterson, *et al.* (3), in their fractionation studies on arabogalactan, prepared the acetyl derivative from an ether suspension of arabogalactan peptized into pyridine by heating with acetic anhydride at 80–85°. White (4), in his studies on the constitution of arabogalactan, prepared the acetyl ester using arabogalactan peptized into pyridine and acetic acid. In an alternate method he boiled arabogalactan with acetic anhydride using sodium acetate as the catalyst. A different method of acetylation is described which yields reproducible results and may be more adaptable for large runs.

Several workers have reported the methylation of arabogalactan (3–5). However, the objective of their work was to bring about complete methylation in order to elucidate the structure of arabogalactan. The objective here was to obtain a partially methylated product which would show an increase in the surfactant property of arabogalactan. This objective has been achieved. Further detailed reports on these and other chemical modifications of arabogalactan and their uses will be presented in a subsequent paper.

EXPERIMENTAL

Use in Tableting

Lactose granulations and lactose-calcium phosphase (dibasic) granulations were made with arabogalactan or acacia solutions as the binder, dried, passed through a No. 16 screen, lubricated with 10% starch and 0.5% magnesium stearate, and compressed into tablets on a Stokes B-2 rotary machine using $^3/_8$ inch standard concave punches. The composition and hardness of each batch is shown in Table I.

Batches 1–6 were compressed to an average thickness of 5.985 mm. (±0.06%) and an average weight of 500 mg. (±1.6%); and batches 7–12 were compressed to an average thickness of 6.042 mm. (±0.06%) and an average weight of 606 mg. (±1.6%). The same pressure setting on the tablet machine was used in all runs.

The hardness of the tablets were then measured using a Strong-Cobb hardness tester. Samples of the tablets were kept at 50, 70, and 80% humidity levels at room temperature (21°), and after a period of three months their physical appearance was compared with those kept in sealed containers protected from moisture. Tablets of riboflavin, folic acid, ascorbic acid, menadione, and aspirin made with arabogalactan and with acacia as binders

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TABLE I.—COMPOSITION AND HARDNESS OF VARIOUS BATCHES OF TABLETS

Batch No.	Binding Solution ml.	Binding Solution w/v	Tablet Hardness[a] (Av. of 10 Tablets ±5%)
1[b]	260	10% arabogalactan	5.9
2[b]	"	15% arabogalactan	6.2
3[b]	"	20% arabogalactan	6.3
4[b]	"	25% arabogalactan	6.4
5[b]	"	33 1/3% arabogalactan	8.0
6[b]	"	10% acacia	7.8
7[c]	300	10% arabogalactan	12.5
8[c]	"	15% arabogalactan	12.7
9[c]	"	20% arabogalactan	12.0
10[c]	"	25% arabogalactan	12.8
11[c]	"	33 1/3% arabogalactan	15.5
12[c]	"	10% acacia	12.7

[a] Strong-Cobb dial reading. [b] Three pounds lactose. [c] One and one-half pounds each of lactose and calcium phosphate.

were kept at room temperature and at 60° and were analyzed weekly for a period of eight weeks for a comparative stability study to establish if arabogalactan had any deteriorating action on the active ingredients. The tablets were made and analyzed as follows:

Riboflavin Tablets.—Lactose was wet granulated using a 20% w/v arabogalactan solution. The granules (16 mesh) were lubricated with 0.75% magnesium stearate. The riboflavin was dry mixed with the lubricated granules and compressed into tablets containing 5 mg. riboflavin per tablet. Another batch of similar riboflavin tablets was made using a 10% w/v solution of acacia as the binder. The U. S. P. XV (6) fluorometric assay was followed using a Coleman electrofluorometer.

Folic Acid Tablets.—Lactose was granulated as above with 20% w/v arabogalactan. The dried granules (16 mesh) were lubricated with 2% talc and 0.75% magnesium stearate, dry mixed with folic acid, and compressed into tablets containing 5 mg. folic acid per tablet. Another batch of similar folic acid tablets was made using 10% acacia as the binder. The spectrophotometric method of analysis, U. S. P. XV (6) was followed using a Beckman spectrophotometer.

Menadione Tablets.—Lactose was granulated with 20% arabogalactan. The dried granules (16 mesh) were lubricated with 2% talc and 0.75% magnesium stearate, dry mixed with the menadione, and compressed into tablets containing 2 mg. menadione per tablet. Another batch of similar menadione tablets was made using 10% acacia as the binder. The U. S. P. XV (6) assay was used.

Ascorbic Acid Tablets.—A mixture of lactose and ascorbic acid was wet granulated with 20% arabogalactan. The granules were dried overnight at 50° and the dried granules (16 mesh) were lubricated with 2% talc and 0.75% magnesium stearate and compressed into tablets containing 100 mg. ascorbic acid per tablet. Another batch of similar ascorbic acid tablets was made using 10% acacia as the binder. The U. S. P. XV (6) method of analysis was used.

Aspirin Tablets.—Powdered aspirin was mixed with 5% starch and wet granulated with 20% arabogalactan, and a similar mixture granulated with 10% acacia. The dried granules (16 mesh) were lubricated with 5% starch and 2% talc and compressed into tablets containing 320 mg. aspirin per tablet. The method of analysis was an adaptation of the method of Pankratz and Bandelin (7). Twenty tablets were used for each assay.

Preparation of Arabogalactan Acetate

Sodium acetate was fused according to the method of Vogel (8). Arabogalactan was powdered to 150 mesh in a homoloid mill so that it could be uniformly suspended, obviating the necessity for peptizing, and dried at 100° for four hours. Seventy grams of the dried finely powdered arabogalactan was triturated with 10 Gm. of fused sodium acetate and 5 Gm. of anhydrous zinc chloride. The mixture was placed in a 3-necked, 1-liter, round-bottomed flask fitted with a vibrostirrer, condenser, and thermometer. Three hundred milliliters of acetic anhydride was added, the mixture stirred for two to three hours, and left overnight for swelling. A further 200 ml. of acetic anhydride was added and the flask was then heated to 130–135° for a period of five hours, with stirring. Then the mixture was allowed to cool to about 50° and the unreacted arabogalactan and catalysts filtered off. The filtrate was poured in a thin stream into a large excess of distilled water and crushed ice. The acetate settled down as a semisolid brown layer, and the contents of the beaker were allowed to sit overnight in the refrigerator. The supernatant liquid was drained off, 50 ml. of acetone was added to the brown liquid, and the mixture was stirred and poured, drop by drop, into crushed ice and distilled water with rapid stirring. The acetate was formed as a light brown precipitate which was filtered, and washed free of acid with distilled water. The above procedure of dissolving in acetone and reprecipitating with water was repeated once again. The resulting product was washed with ether, powdered, and dried at 80° for six hours. Yield, 63 Gm.

Purification.—The above material was dissolved in about 1 L. of alcohol by boiling and 3 Gm. of Norite was added and stirred. One hundred milliliters of acetone was added to dissolve the ester which separates when the alcoholic solution is cooled. The Norite was filtered off and the solution concentrated to one-fourth its volume and poured in a thin stream into iced water, with stirring. The precipitate was filtered off (sometimes it may be necessary to concentrate the liquid to obtain the precipitate), washed with water, then ether; powdered, and dried. The product obtained was an off-white powder with a yellow tinge. It melted between 176–182°. The I. R. spectrum showed a strong absorption band at 1,745 $cm.^{-1}$ characteristic of saturated aliphatic esters. It was soluble in acetone and insoluble in ether and water. Yield, 48 Gm.

Reacetylation.—A 5-Gm. sample of arabogalactan acetate was reacetylated following the above pro-

cedure. The product melted between 176–182° and a mixed melting point with arabogalactan acetate prepared above showed no depression. The I. R. spectrum was identical with the previously prepared arabogalactan acetate.

Acetyl Value.—The acetyl value of the ester was determined (9) and was found to be 47.225% w/w of $-COCH_3$. Reacetylation gave essentially the same value (47.230% w/w of $-COCH_3$).

Deacetylation.—A 3-Gm. sample of arabogalactan acetate was dissolved in 30 ml. of acetone and 75 ml. of 2 *N* alcoholic potassium hydroxide was added and stirred at room temperature. There was immediate flocculation, and precipitation resulted soon after. After one-half hour, the precipitate was filtered off, washed successively with 2% alcoholic acetic acid, ethanol, ether, and then dried. Yield, 1.9 Gm.

The I. R. spectrum compared closely with starting arabogalactan indicating that no major degradative changes have occurred during acetylation and saponification. However, in the I. R. spectrum of the deacetylated compound, a strong absorption band appears at 1,485 cm.$^{-1}$ and the small peak at 1,645 cm.$^{-1}$ in the original arabogalactan appears as a shoulder.

Preparation of Methyl Arabogalactan

A 50-Gm. sample of arabogalactan was dissolved in 50 ml. water and placed in a three-necked flask fitted with a condenser, stirrer, and dropping tube. A mixture of 90 ml. of methyl sulfate in 125 ml. of carbon tetrachloride was added rapidly through the dropping tube. Four hundred milliliters of 60% sodium hydroxide was placed in the reservoir and added to the flask at the rate of three drops every second until the distillation of carbon tetrachloride slackened and ceased. (Usually accomplished in twenty minutes after the addition of 70 to 90 ml. of alkali.) Continuous stirring was necessary throughout the reaction. The remainder of the alkali was added to the flask as quickly as possible. Methyl sulfate, 160 ml., was placed in the reservoir and added three to four drops per second (slower if mixture foams) after which the contents of the flask were cooled, and the separated product removed by filtration. It was then dissolved in chloroform and precipitated by petroleum ether. It was purified by repeatedly redissolving in chloroform and reprecipitating with petroleum ether. The product was found to be reproducible by comparing the I. R. spectra of six different batches. The product is an off-white solid of m. p. range 154–169°. Mixed melting points show no depression. It is soluble in water and chloroform but insoluble in ether and petroleum ether. Yield, 18 Gm.

Methoxyl Content.—The methoxyl content of the product is 33.4-35.2%. The methoxyl content of the fully methylated compound has been reported as 44.1 (3), 44.3 (4), and 44.4% (5).

RESULTS, DISCUSSION, AND APPLICATION

Use in Tableting

The binding capacity of arabogalactan as indicated by tablet hardness is shown in Table I and Fig. 1. A higher concentration of arabogalactan than acacia seems necessary to give the same binding effect. It should be noted however that the above tablets could have been made harder by using greater pressure during the compression. As a Strong-Cobb hardness of 7 to 8 is the normal criteria for tablet hardness for many types of tablets, it is suggested that 20% w/v arabogalactan solution would be a very satisfactory binder. Arabogalactan did not hamper the disintegration time. The 12 batches of tablets disintegrated within six minutes using the U. S. P. XV (6) disintegration test.

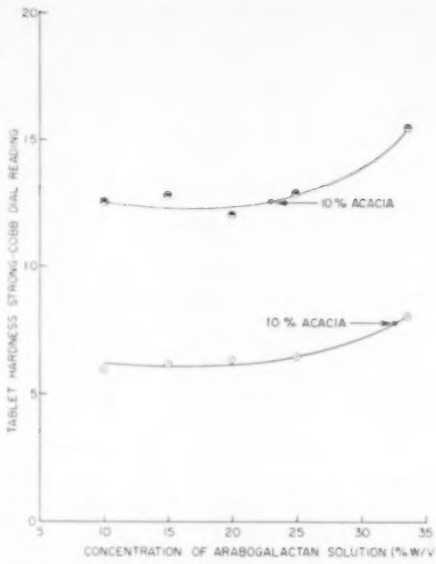

Fig. 1.—Tablet hardness vs. concentration of binding solution. ●—●, lactose-calcium phosphate tablets; ○—○, lactose tablets.

The appearance of shelf-stored samples and those at various humidity levels at 21° after a three-month aging period is shown in Table II.

Nonalkaline tablets made with arabogalactan did not undergo any visible physical changes, but tablets that may have an alkaline pH showed some instability especially at the higher humidity levels. This alkaline instability is probably due to some phenolic impurities in arabogalactan used in these

Table II.—Appearance (after Three Months) of Tablets Made with Arabogalactan and Acacia as a Binder[a]

Batch No.	Shelf	Humidity at 21° 50%	70%	80%
1	A	A	A	A
2	A	A	A	A
3	A	A	A	A
4	A	A	A	A
5	A	A	A	A
6	A	A	A	A
7	A	A	NA	NA
8	A	A	NA	NA
9	NA	NA	NA	NA
10	NA	NA	NA	NA
11	NA	NA	NA	NA
12	A	A	NA	NA

[a] A, acceptable; NA, not acceptable.

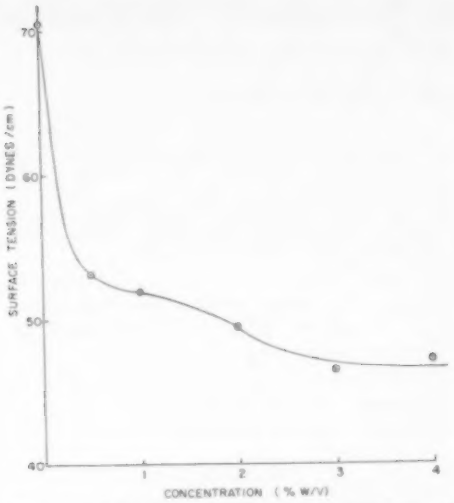

Fig. 2.—Surface tension vs. concentration of partially methylated arabogalactan at 20 ± 0.5°.

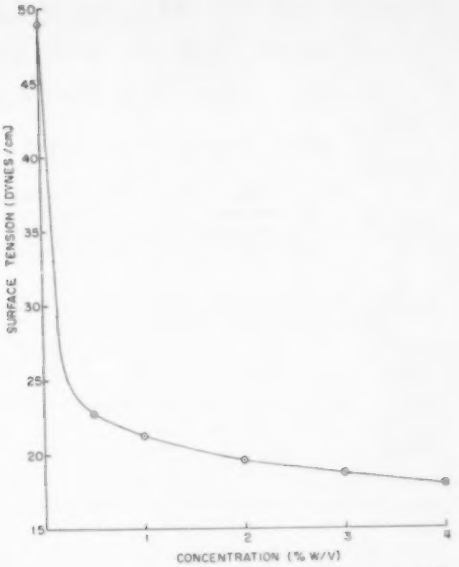

Fig. 3.—Interfacial tension between partially methylated arabogalactan solution and liquid petrolatum vs. concentration at 20 ± 0.5°.

studies (10); a purer material devoid of phenolic impurities is being experimented with in our laboratories and it shows very good promise in that alkalies have no deteriorating effect on it. It is anticipated that this purer material will eventually be available in larger quantities.

Arabogalactan did not cause any more deterioration than acacia in tablets of riboflavin, folic acid, menadione, ascorbic acid, and aspirin when analyzed weekly over an eight-week aging period at room temperature and 60°, as seen in Table III.

Arabogalactan Acetate.—The uses and toxicity of arabogalactan acetate are being studied. Preliminary work indicates that it is an excellent film former and shows considerable promise in film coating of tablets. This and related aspects now under study will be reported in a subsequent paper.

Methyl Arabogalactan.—Applications and uses of this product are under study. Preliminary investigation shows that it possesses surfactant properties (Figs. 2 and 3) which are greater than those of arabogalactan (1).

TABLE III.—FINAL ANALYSIS VALUES AFTER AN EIGHT-WEEK AGING PERIOD TAKING THE INITIAL ANALYSIS TO BE 100%

	With Arabogalactan, %		With Acacia, %	
Type of Tablet	Room Temp.	60°	Room Temp.	60°
Riboflavin	98.8	93.2	97.7	91.8
Folic acid	98.9	98.7	98.3	98.0
Menadione	100.0	94.0	100.0	93.9
Ascorbic acid	99.6	97.5	99.8	97.4
Aspirin	100.0	99.1	100.0	99.0

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Inhibition of Mitochondrial Monoamine Oxidase of Canine Spleen and Liver by 1-Isonicotinyl-2-isopropylhydrazine (Iproniazid)

By PRABHAKER D. LOTLIKAR† and ROB S. McCUTCHEON

The inhibitory effects of 1-isonicotinyl-2-isopropylhydrazine (iproniazid) on monoamine oxidase of dog liver is well known. This investigation sought to determine the monoamine oxidase inhibitory effect of this drug in dog spleen when injected intramuscularly and when added to spleen mitochondria *in vitro*.

MONOAMINE OXIDASE (MO) activity in the spleen of pig, ox, and sheep was first observed by Bhagwat (1), who reported that the specific activity in this tissue was less than 10 per cent of that in liver and kidney. Among the inhibitors of monoamine oxidase activity of several mammalian tissues, iproniazid has been found to be a potent member (2). The monoamine oxidase activity of mitochondria prepared from hog kidney cortex, and from liver or brain of mouse, rabbit, cat, dog, ox, and man, was found by Zeller (2) to be inhibited from 90 to 100 per cent by 10^{-4} *M* iproniazid. Previous studies have shown that administration to dogs of 0.4 m*M* of iproniazid per Kg. of body weight caused a complete inactivation of monoamine oxidase activity in liver homogenates (2). It was also shown that preincubation of liver monoamine oxidase with iproniazid was necessary to obtain maximum inhibition (2). Davison (3) and Gluckman (4) have shown that this inhibition is easily prevented by the further addition of substrate. Davison (3) also observed that monoamine oxidase activity of liver mitochondria was much increased in the presence of potassium cyanide. Corne (5) has reported that iproniazid in a dose of 10 to 20 mg./Kg. is completely effective two hours after injection in inhibiting amine oxidase activity of liver and kidney homogenates of rat, cat, and guinea pig.

Epinephrine and norepinephrine both provoke constriction of the spleen, increasing the circulating blood volume and erythrocyte count. Since it has been postulated that both these are inactivated by monoamine oxidase, it was felt important to determine the monoamine oxidase activity of canine spleen and the inhibiting action, if any, of iproniazid on this organ preliminary to a study of splenic adrenergic receptors and the interaction of epinephrine and iproniazid. For comparative purposes, like experiments were run on dog liver.

EXPERIMENTAL

Hess (6) has shown that monoamine oxidase inhibition was complete in five or six days after intraperitoneal administration of iproniazid to rats. Thus, in this study, animals were given iproniazid 50 μM/Kg. body weight per day intramuscularly for eight days and then were tested for monoamine oxidase activity of spleen and liver mitochondria. These results were compared with those from the liver and spleen of untreated animals, in which iproniazid was added to mitochrondria and compared with monoamine oxidase activity of mitochondria alone.

Mongrel dogs of either sex weighing between 10–15 Kg. were used in this study. The animals were anesthetized by intraperitoneal injection of pentobarbital (35 mg./Kg.) and the liver and spleen removed.

Mitochondrial Preparations.—The tissue was finely cut and suspended in 100 ml. of 0.25 *M* sucrose. It was then homogenized in a Waring Blendor during four short periods (one, one, one, five seconds). An average of 30 Gm. of spleen and 20 Gm. of liver was used for separate homogenates in each case. The homogenate was passed through eight layers of cheesecloth. The filtrate was then centrifuged at 600 × g for ten minutes to sediment cells and debris. The mitochondria were brought down at 10,000 × g and then washed twice with glass distilled water and suspended in distilled water.

Oxygen Uptake.—Warburg respirometers were used to measure oxygen uptake at 37.5°. The composition of the reaction mixture was according to Creasey (7).

Main Compartment.—Enzyme preparation (1.0 ml.); 0.24 *M* sodium phosphate buffer (0.3 ml.); 0.01 *M* potassium cyanide (0.3 ml.); 0.1 *M* semicarbazide (0.3 ml.); iproniazid 5×10^{-3} *M* or 5×10^{-2} *M* (0.6 ml.) as indicated; distilled water as necessary.

Side Arm.—Tyramine, 0.1 *M* (0.3 ml.). Total volume of liquid medium in the main compartment and side arm was 3.0 ml.

Center Well.—Potassium cyanide, 2 *M* (0.1 ml.); and filter paper strip. All solutions except 2 *M* potassium cyanide were adjusted to pH 7.0.

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The iproniazid used in this study was kindly furnished by the research department of Hoffmann-La Roche, Inc., as Marsilid phosphate.

TABLE I.—STUDIES OF MONOAMINE OXIDASE ACTIVITY OF SPLEEN AND LIVER MITOCHONDRIA FROM UNTREATED DOGS

	$Q_{O_2}^{O_2}$ (N)[a]	No.[b]	S. D.[c]
Part A.—MO Activity of Spleen Mitochondria			
1. Untreated tissue	23.3	6	±6.38
2. Untreated tissue + iproniazid 1×10^{-3} *M*	6.1	3	±1.40
3. Untreated tissue + iproniazid 1×10^{-2} *M*	9.7	4	±3.06
Concentration of spleen mitochondrial nitrogen, 3–6 mg./ml.			
Part B.—MO Activity of Liver Mitochondria			
1. Untreated tissue	155.8	7	±25.95
2. Untreated tissue + iproniazid 1×10^{-3} *M*	15.9	6	±12.49
3. Untreated tissue + iproniazid 1×10^{-2} *M*	0.0	6	.....
Concentration of liver mitochondrial nitrogen, 1.5–3.0 mg./ml.			

[a] $Q_{O_2}^{O_2}$ (N), Average values in μl. of oxygen uptake per mg. of mitochondrial nitrogen per hour in an atmosphere of pure oxygen.
[b] No., number of experiments.
[c] S. D., Standard Deviation. S. D. = $\sqrt{\Sigma(Y - Y)^2/(N - 1)}$.

ANALYSIS OF VARIANCE FOR TABLE I, PART A

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Treatment	761.96	2	380.98	16.32
(*a*) 1 *vs.* 3	442.27	1	442.27	18.94
(*b*) 2 *vs.* 3	22.53	1	22.53	0.96
Error	233.49	10	23.35	
Total	995.45	12		

Conclusion: (*a*) The means of treatments 1 and 3 are significantly different. (*b*) The means of treatments 2 and 3 are not significantly different.

TABLE OF MEANS

Treatment	1	2	3
Number	6	3	4
Mean	23.300	6.100	9.725

ANALYSIS OF VARIANCE FOR TABLE I, PART B

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Treatment	160,612.37	1	160,612.37	384.66
Error	4,593.00	11	417.55	
Total	165,205.37	12		

Conclusion: The treatment means are significantly different.

TABLE OF MEANS

Treatment	1	2
Number	7	6
Mean	155.800	15.900

The flasks were perfused with oxygen for five minutes and equilibrated for ten minutes before tipping in the substrate. Oxygen uptake was recorded zero, thirty, sixty, ninety, and one hundred and twenty minutes. In both the spleen and liver preparations, the activity dropped off after 30 minutes of incubation. Therefore, only the thirty-minute readings were used for the determinations of $Q_{O_2}^{O_2}$ (N) values.

Determinations of mitochondrial nitrogen were carried out by the micro-Kjeldahl method (8).

Chemicals.—Tyramine hydrochloride, from Nutritional Biochemical Corp., Cleveland, Ohio, and used without further purification. Other chemicals used were of reagent grade.

RESULTS AND DISCUSSION

Part A of Table I shows that in the *in vitro* studies, iproniazid at 1×10^{-3} *M* apparently causes slightly more inhibition of spleen monoamine oxidase activity than it does at 1×10^{-2} *M*. Our analysis of variance did not confirm this. However, it may be seen in part B that with monoamine oxidase of liver mitochondria, iproniazid at 1×10^{-3} *M* inhibits 90% of the activity and at 1×10^{-2} *M* iproniazid inhibits monoamine oxidase activity completely.

Table II shows the levels of monoamine oxidase activity of spleen and liver mitochondria of dogs after administration of 50 μ*M* iproniazid per Kg. body weight per day for eight days. In these experiments iproniazid was also added to the Warburg flasks to observe its effect on the residual activity.

It can be seen that spleen mitochrondria, after the dogs had been on iproniazid for eight days, had an appreciable monoamine oxidase activity, which equalled about 70% of that found in spleen mitochondria from control (untreated) animals. This residual activity is further decreased by the *in vitro* addition of iproniazid at 1×10^{-3} *M* and seems to be partially restored by iproniazid at 1×10^{-2} *M*. This last effect was not confirmed by the analysis of variance.

The table also shows that liver mitochondrial monoamine oxidase activity is completely destroyed by *in vivo* administration of the inhibitor and further addition of iproniazid did not have any effect.

The studies show that monoamine oxidase activity in liver mitochondria in previously untreated animals is almost completely inhibited by 1×10^{-3} *M* iproniazid. After 50 μ*M* iproniazid per Kg. body weight per day for eight days is administered to the intact dog, the monoamine oxidase activity of liver is zero, confirming the results of Zeller (2).

Monoamine oxidase activity in spleen mitochondria is comparatively less than that of liver mitochondria. However, spleen monoamine oxidase is less sensitive to iproniazid. The results with spleen monoamine oxidase obtained with preparations from treated and untreated animals, indicate that its activity may be more at higher concentrations of inhibitor, 1×10^{-2} *M* than at the lower concentration of 1×10^{-3} *M*. However, confirmation of this must await further experimentation. It may be that the apparent reversal of monoamine oxidase

TABLE II.—STUDIES OF MONOAMINE OXIDASE ACTIVITY OF SPLEEN AND LIVER MITOCHONDRIA FROM DOGS AFTER ADMINISTRATION OF IPRONIAZID EIGHT DAYS TO INTACT ANIMAL

	$Q_{O_2}^{O_2}$ (N)[a]	No.[b]	S. D.[c]
Part A.—MO Activity of Spleen Mitochondria			
1. Spleen from treated animal	16.4	4	±3.31
2. Spleen from treated animal + iproniazid 1×10^{-3} *M*	7.1	4	±2.15
3. Spleen from treated animal + iproniazid 1×10^{-2} *M*	10.5	4	±4.58
Concentration of spleen mitochondrial nitrogen, 3–6 mg./ml.			
Part B.—MO Activity of Liver Mitochondria			
1. Liver from treated animal	0	6	....
2. Liver from treated animal + iproniazid 1×10^{-3} *M*	0	6	....
3. Liver from treated animal + iproniazid 1×10^{-2} *M*	0	6	....
Concentration of liver mitochondrial nitrogen, 1.5–3.0 mg./ml.			

[a] $Q_{O_2}^{O_2}$ (N), Average values in µl. of oxygen uptake per mg. of mitochondrial nitrogen per hour in an atmosphere of pure oxygen.
[b] No., number of experiments.
[c] S. D., standard deviation.

ANALYSIS OF VARIANCE FOR TABLE II, PART A

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Treatment	176.14	2	88.06	7.13
Error	111.20	9	12.35	
Total	287.33			

Conclusion: The treatment means are significantly different.

TABLE OF MEANS

Treatment	1	2	3
Number	4	4	4
Mean	16.400	7.125	10.525

The least significant difference between two means, at 5% level, is 5.621.

The mean of treatment 1 is significantly higher than those of treatments 2 and 3. The difference between the means of treatments 2 and 3 is not significant.

inhibition in the spleen by increased concentrations of iproniazid is the result of dehydrogenation of iproniazid at the active center of the enzyme which may account for an increase in oxygen uptake at the higher concentration. Thus, the oxidation of iproniazid at the higher concentration may be greater than at the lower. The lower inhibition of spleen monoamine oxidase compared to liver monoamine oxidase may be due to different properties of the enzyme or may depend upon some protective factors in the mitochondrial preparations. In addition, spleen may contain extra mitochondrial [illegible] which protect monoamine oxidase against inhibition when iproniazid is administered to intact dogs.

SUMMARY

1. It has been shown that after the administration to dogs of 50 µ*M* iproniazid/Kg. body weight/day for eight days, the spleen mitochondria have a monoamine oxidase activity of 70 per cent that of untreated animals.

2. The results with spleen mitochondrial monoamine oxidase, comparing treated and untreated animal spleen, indicate the possibility that the activity is greater at higher concentration of inhibitor (1×10^{-2} *M*) than at lower concentrations (1×10^{-3} *M*).

3. The liver mitochondrial monoamine oxidase activity of treated animals is completely inhibited, that from untreated animals is completely inhibited by the 1×10^{-2} *M* concentration of iproniazid.

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Preparation and Properties of New Gastric Antacids III

Aluminum Hydroxide-Glycine

By STEWART M. BEEKMAN

The preparation and properties of two new, highly reactive aluminum hydroxide-glycine (AHG) gels and dried gels are described. The rate of reaction with gastric acid is shown to be rapid and prolonged in the optimum pH range of 3 to 5. Four methods were used to determine reaction rate including Reheis and Mutch reaction velocity tests; modified procedure of Holbert, Noble, and Grote; and the method of Fuchs. The latter two were carried out by means of an automated apparatus for antacid evaluation described in the first paper of this series. The *in vitro* antacid properties of aluminum hydroxide-glycine dried gels compare favorably with reactive liquid aluminum hydroxide gel. Aging for seven years has not lessened the reactivity of the dried gels. A modified aluminum hydroxide-glycine dried gel containing substantial amounts of magnesium hydroxide is shown to yield outstanding antacid activity in the pH range of 3 to 4.5.

THE PURPOSE of this paper is to describe two new aluminum hydroxide-glycine (AHG) antacids in gel and dried gel form and to report the results of their evaluation using several *in vitro* methods. They are designated as types F-4A and F-4B and vary principally in the mole ratio of alumina (aluminum oxide) to glycine, which is 0.5:1.0 and 1:1, respectively.

The use of glycine in antacids is not new. In 1944 Krantz (1) and his associates reported the preparation of an interesting new aluminum salt of glycine, dihydroxy aluminum aminoacetate. The outstanding antacid activity of this compound has been reported by many investigators (2–11). In 1947 Abramson (12) added three parts of glycine to seven parts of calcium carbonate to prepare an antacid which was alleged to have a buffering action similar to that obtained from fresh milk. Also in 1947, Gjaldbaek (13) stated that glycine is effective in reducing hydrogen ion concentration, is very suitable with respect to secondary secretion, brings about a small change in osmotic pressure, but does not reduce the titration acidity.

The aluminum hydroxide-glycine gels (AHG), which are the subject of this paper, are smooth, white, stable, homogeneous hydrogels of aluminum. They form flowable, thixotropic liquid preparations in concentrations up to 8% alumina, which is double the U. S. P. strength for aluminum hydroxide gel. They are prepared by dispersing glycine crystals in a highly reactive and stable aluminum hydroxide gel at room temperature. In addition to being rapidly and completely reactive with gastric strength acid, the gels have a bland, mildly sweet, pleasant taste. Table I shows the composition and some properties of F-4A and F-4B aluminum hydroxide-glycine compressed gels.

Aluminum hydroxide-glycine dried gels are made by careful desiccation of the wet gels using spray drying or low temperature air drying followed by reduction to fine particle size. The resulting white, impalpable powder is rapidly reactive with gastric strength acid containing pepsin. It also maintains this reactivity for many years, aging at ambient temperatures. The bland tasting, slightly sweet powder readily rehydrates and swells when added to water. Tablets made therefrom have a smooth, pleasant nonchalky taste property.

In Table II typical analyses and some of the antacid properties of aluminum hydroxide-glycine dried gels types F-4A and F-4B are compared with those of aluminum dihydroxy aminoacetate and aluminum hydroxide dried gel U. S. P.

TABLE I.—COMPOSITION OF TYPICAL SAMPLES OF ALUMINUM HYDROXIDE-GLYCINE COMPRESSED GELS

Entity	Type F-4A	Type F-4B
Aluminum oxide (Al_2O_3), %	8.7	9.3
Glycine, %	7.8	6.9
Carbonate (as CO_2), %	3.7	4.0
Al_2O_3:glycine, mole ratio	0.5:1	1:1
pH of 4.0% Al_2O_3 suspension	6.8	6.8
Specific gravity	1.05	1.05
Acid consuming capacity (ml. 0.1 *N* HCl per Gm.)	50	54
Reaction velocity		
Reheis method sec.	65	45
Mutch method sec.	65	45
Viscosity of hydrogels cps.		
4% Al_2O_3	107	68
8% Al_2O_3	1020	1140

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TABLE II.—COMPARISON OF TYPICAL ANALYSES OF DRIED GELS

Entity	Aluminum Hydroxide-Glycine Dried Gel Type F-4A	Aluminum Hydroxide-Glycine Dried Gel Type F-4B	Aluminum Hydroxide Dried Gel U. S. P.	Aluminum Dihydroxy Aminoacetate
Al_2O_3, %	32.5	37.5	51.0	33.7
Glycine, %	47.7	26.8	...	48.6
Carbonates (CO_2), %	5.3	9.7	9.4	...
Water (Karl Fischer), %	...	15.7	22	17.5
Al_2O_3:glycine mole ratio (theoretical)	0.5:1	1:1	...	0.5:1
pH 4% aqueous dispersion	7.5	7.4	8.3	7.2
Acid consuming capacity (ml. 0.1 *N* HCl per Gm.)	188	222	280	185
Acid consuming capacity (ml. 0.1 *N* HCl per Gm. Al_2O_3)	554	582	550	549
Reaction velocity				
Reheis method sec.	15	15	240	29
Mutch method sec.	50	40	1080	103

EXPERIMENTAL

X-ray Diffraction Studies.—X-ray diffraction studies were made using the Debye-Scherrer powder technique with thin-wall glass capillaries (0.300 mm. o. d. and 0.250 mm. i. d.). The exposures were made for three hours at 30 kv. and 25 ma. on the X-ray tube. Samples tested were aluminum hydroxide-glycine dried gel type F-4B, aluminum hydroxide dried gel, aminoacetic acid N. F., and aluminum dihydroxy aminoacetate. The latter gave a distinct pattern with its strongest line at 12.04 Å. and evidenced a high degree of crystallinity. The aminoacetic acid gave a distinct diagram showing a high degree of crystallinity. Both the aluminum hydroxide-glycine type F-4B and the dried aluminum hydroxide from which it was made gave no pattern, indicating that they are essentially amorphous. Although the glycine gave a distinct pattern, the material lost its apparent crystallinity when made into the F-4B mixture.

Infrared Spectra.—Rock salt infrared spectra (2 to 15 μ) were made on samples of aluminum hydroxide-glycine dried gel type F-4B as well as each component separately dried. Nujol as well as hexachlorobutadiene (HCBD) mulls were made. A careful examination of the three spectra showed that the absorption bands of the two components were contained in the spectra of AHG dried gel type F-4B and also that it is predominantly a simple mixture of aluminum hydroxide and glycine and not a new chemical compound.

Antacid Activity.—Five *in vitro* methods were used to evaluate the antacid activity of these new antacids as follows: (*a*) acid consuming capacity test, (*b*) Reheis reaction velocity test, (*c*) Mutch reaction velocity test, (*d*) procedure of Holbert, Noble, and Grote modified, and (*e*) method of Fuchs.

The details of the first four methods are contained in the first paper (11) of this series of studies. Values for the first three tests are found in Table I for the wet gels and in Table II for the dried gels.

Procedure of Holbert, Noble, and Grote Modified.—This method is fully described in a previous paper (11). Results obtained on samples of AHG gels and dried gels, aluminum hydroxide gel U. S. P., aluminum hydroxide dried gel U. S. P., and aluminum dihydroxy aminoacetate are shown in Table III. The results on AHG gels and dried gels are also plotted in Figs. 1–5.

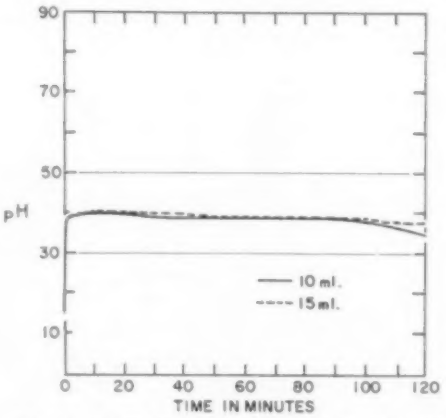

Fig. 1.—Aluminum hydroxide-glycine gel type F-4A 4% aluminum oxide. Procedure of Holbert, Noble, and Grote modified.

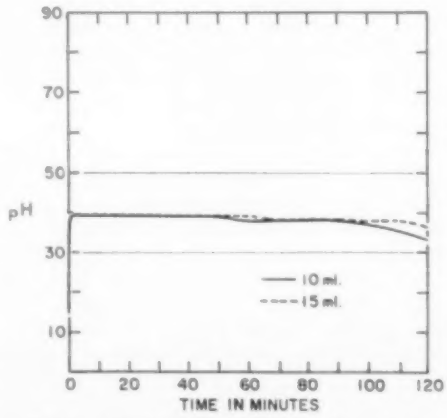

Fig. 2.—Aluminum hydroxide-glycine gel type F-4B 4% aluminum oxide. Procedure of Holbert, Noble, and Grote modified.

TABLE III.—*In Vitro* EVALUATION OF ANTACID ACTIVITY OF ALUMINUM HYDROXIDE-GLYCINE ANTACIDS[a]

Time, min.	Aluminum Hydroxide-Glycine Gel—Type F-4A—4% Al_2O_3 10 ml.	15 ml.	8% Al_2O_3 3 ml.	5 ml.	—Type F-4B—4% Al_2O_3 10 ml.	15 ml.	8% Al_2O_3 3 ml.	5 ml.	Aluminum Hydroxide Gel U. S. P. 5 ml.	10 ml.	15 ml.	Aluminum Hydroxide-Glycine Dried Gel—Type F-4A— 0.5 Gm.	1.0 Gm.	2.0 Gm.	—Type F-4B— 0.5 Gm.	1.0 Gm.	2.0 Gm.	Aluminum Hydroxide Dried Gel U. S. P. 1.0 Gm.	2.0 Gm.	Aluminum Dihydroxy Aminoacetate 0.5 Gm.	1.0 Gm.	2.0 Gm.
0	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
1	3.1	3.6	3.0	3.0	3.4	3.6	2.8	3.4	2.6	3.8	3.9	2.5	3.7	4.1	2.2	3.9	3.6	2.0	2.2	2.1	3.0	3.8
2	3.7	3.8	3.8	3.6	3.8	3.8	3.8	3.8	3.8	3.9	4.0	3.1	3.9	4.2	3.4	4.0	3.9	2.0	2.3	2.7	3.7	4.0
3	3.9	3.9	3.9	3.8	3.9	3.9	3.9	3.9	3.9	3.9	4.0	3.5	4.0	4.2	3.6	4.0	4.0	2.1	2.4	3.2	3.9	4.1
4	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	4.1	3.7	4.0	4.2	3.7	4.0	4.0	2.1	2.4	3.4	3.9	4.1
5	3.9	4.0	3.9	3.9	3.9	4.0	3.9	4.0	3.9	3.9	4.1	3.8	4.0	4.2	3.7	4.0	4.0	2.1	2.5	3.6	4.0	4.1
10	4.0	4.0	3.9	3.9	3.9	4.0	3.9	4.0	3.9	3.9	4.2	3.9	4.0	4.2	3.9	4.0	4.1	2.3	3.3	4.0	4.2	4.2
20	4.0	4.0	3.9	3.9	3.9	4.0	3.9	4.0	3.9	3.9	4.2	3.8	4.0	4.2	3.8	4.0	4.1	2.9	4.0	4.0	4.2	4.2
30	3.9	4.0	3.9	4.0	3.9	4.0	3.9	4.0	3.9	3.9	4.2	3.8	4.0	4.2	3.8	4.0	4.1	3.9	4.0	4.0	4.1	4.2
40	3.9	3.9	3.9	3.9	3.9	3.9	3.9	4.0	3.9	3.9	4.2	3.8	4.0	4.2	3.8	4.0	4.0	3.9	4.0	3.8	4.1	4.2
50	3.9	3.9	3.9	3.9	3.9	3.9	3.9	4.0	3.9	3.9	4.2	3.5	3.9	4.1	3.7	4.0	4.0	3.8	4.0	3.6	4.0	4.2
60	3.9	3.9	3.8	3.9	3.8	3.9	3.8	4.0	3.8	3.9	4.1	3.2	3.9	4.1	3.3	4.0	4.0	3.8	4.0	3.3	4.0	4.2
70	3.9	3.9	3.7	3.9	3.8	3.9	3.7	3.9	3.5	3.9	4.1	2.8	3.8	4.0	2.9	3.9	4.0	3.7	4.0	3.0	3.9	4.2
80	3.9	3.9	3.5	3.9	3.8	3.9	3.5	3.9	3.0	3.9	4.1	2.6	3.7	4.0	2.7	3.9	4.0	3.5	3.9	2.8	3.8	4.2
90	3.9	3.9	3.1	3.8	3.8	3.9	3.1	3.9	2.6	3.8	4.1	2.5	3.5	3.9	2.5	3.9	4.0	3.2	3.9	2.7	3.6	4.1
100	3.8	3.9	2.8	3.7	3.7	3.9	2.7	3.8	2.4	3.8	4.1	2.4	3.3	3.9	2.4	3.8	3.9	3.0	3.9	2.5	3.4	4.1
110	3.7	3.8	2.7	3.5	3.6	3.8	2.6	3.5	2.2	3.7	4.1	2.3	3.0	3.9	2.3	3.7	3.9	2.8	3.8	2.4	3.2	4.1
120	3.5	3.8	2.5	3.2	3.3	3.7	2.5	3.2	2.1	3.4	4.1	2.2	2.8	3.9	2.2	3.5	3.9	2.6	3.7	2.3	3.0	4.0
130	3.1	3.8	2 3	2.9	2.9	3.7	...	2.9	...	2.7	4.1	...	...	3.7	...	3.2	3.9	...	...	...	...	...
140	2.8	3.5	2.2	2.8	2.7	3.5	...	2.7	...	2.5	4.0	...	...	3.5	...	3.0	3.9	...	...	...	...	...
150	2.7	3.3	2.1	2.6	2.5	3.2	...	2.6	...	...	4.0	...	...	3.3	...	...	3.8	...	...	...	...	...
160	...	3.0	...	2 5	...	...	...	2.4	...	...	3.9	...	...	3.0	...	...	3.7	...	...	...	...	...
170	...	2.8	...	2.3	...	...	...	2.3	...	...	3.6	...	...	2.8	...	...	3.4	...	...	...	...	...
180	...	...	...	2.2	...	...	...	2.1	...	...	3.3	...	...	2.7	...	...	3.0	...	...	...	...	...
190	...	...	...	2.0	...	...	...	2.0	...	...	...	...	...	2.6	...	...	...	...	...	...	...	...
200	...	...	...	...	...	...	...	...	...	...	...	...	...	2.4	...	...	...	...	...	...	...	...

[a] pH *versus* time at various dose levels—procedure of Holbert, Noble, and Grote modified.

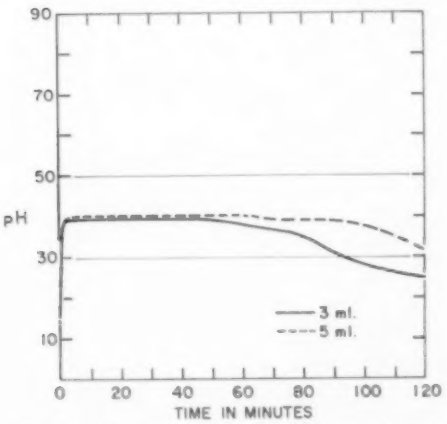

Fig. 3.—Aluminum hydroxide-glycine gel type F-4B 8% aluminum oxide. Procedure of Holbert, Noble, and Grote modified.

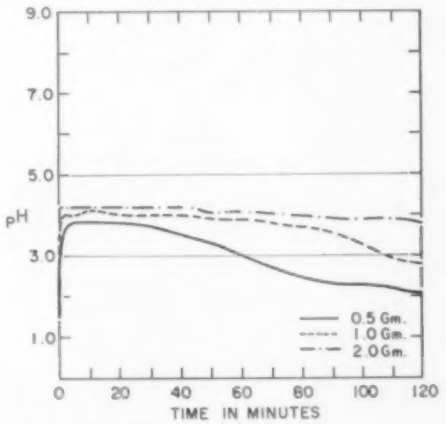

Fig. 4.—Aluminum hydroxide-glycine dried gel type F-4A. Procedure of Holbert, Noble, and Grote modified.

Method of Fuchs.—In 1949 Fuchs (14) in an excellent review of antacids proposed an *in vitro* method for evaluating them. This method, we feel, is very useful although it does not show the inhibition of antacid activity by pepsin. Also, the strength of the replacement acid is high (1 *N*). It has been used by several investigators, including Dale and Booth (15), who published a very comprehensive study of many antacid chemicals and preparations.

Our automated apparatus was adapted to the requirements of this method. One dose of antacid was added to the equivalent of 50 ml. 0.1 *N* hydrochloric acid at 37.5° in a glass reaction cell provided with suitable agitation and pH electrodes. After ten minutes 1.0 *N* hydrochloric acid at 37.5° was added at the equivalent of 12.0 ml. per hour. The pH was continuously recorded as a function of time until the level dropped below 2.5.

Values obtained on 10-ml. samples of aluminum hydroxide gel U. S. P. as well as the new aluminum hydroxide-glycine gels are shown in Table IV. Also

TABLE IV.—*In Vitro* EVALUATION OF ANTACID ACTIVITY OF ALUMINUM HYDROXIDE-GLYCINE ANTACIDS[a]

Time, min.	Aluminum Hydroxide-Glycine Gel Type F-4A, 4% Al_2O_3, 10 ml.	Aluminum Hydroxide-Glycine Gel Type F-4B, 4% Al_2O_3, 10 ml.	Aluminum Hydroxide Gel U. S. P., 10 ml.	Aluminum Hydroxide-Glycine Dried Gel Type F-4A, 1.0 Gm.	Aluminum Hydroxide-Glycine Dried Gel Type F-4B, 1.0 Gm.	Aluminum Dihydroxy Aminoacetate, 1.0 Gm.	Aluminum Hydroxide Dried Gel U. S. P. 1.0 Gm.	Aluminum Hydroxide Dried Gel U. S. P. 2.0 Gm.
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.5	2.9	2.6	3.1	3.3	3.2	2.2	<2.0	<2.0
1	3.1	3.3	3.4	3.5	3.5	3.2	<2.0	<2.0
2	3.5	3.6	3.5	3.6	3.6	3.4	<2.0	<2.0
3	3.7	3.7	3.6	3.7	3.6	3.5	<2.0	<2.0
4	3.8	3.7	3.6	3.7	3.6	3.6	<2.0	<2.0
5	3.9	3.8	3.7	3.7	3.6	3.6	<2.0	<2.0
10	3.9	3.8	3.7	3.7	3.6	3.8	3.1	3.7
20	3.8	3.7	3.6	3.7	3.5	3.6	3.4	3.5
30	3.7	3.7	3.6	3.6	3.4	3.5	3.2	3.5
40	3.7	3.7	3.5	3.5	3.4	3.4	3.2	3.5
50	3.7	3.6	3.5	3.3	3.4	3.1	3.2	3.4
60	3.6	3.6	3.5	3.3	3.4	2.8	3.2	3.4
70	3.5	3.5	3.5	3.0	3.3	2.6	3.1	3.4
80	3.4	3.3	3.4	2.7	3.1	2.3	3.1	3.4
90	3.3	3.1	3.3	2.4	2.8	..	3.0	3.3
100	3.2	2.8	3.2	2.2	2.3	..	2.9	3.3
110	3.0	2.5	2.6	..	..	..	2.7	3.3
120	2.7	2.4	2.1	..	..	..	2.3	3.3
130	2.6	2.3	..	..	..	..	..	3.3
140	2.3	2.1	..	..	..	..	..	3.3
150	2.1	..	..	..	..	..	..	..
Time to pH 3.5 (min.)	2	1.5	2	1	1	3	..	..
Equivalent ml. 0.1 *N* HCl								
to pH 3.0	242	214	242	190	198	140	210	510
to pH 2.5	290	250	252	206	220	170	260	534
to pH 2.0	336	314	274	240	242	204	282	550

[a] pH *versus* time, method of Fuchs.

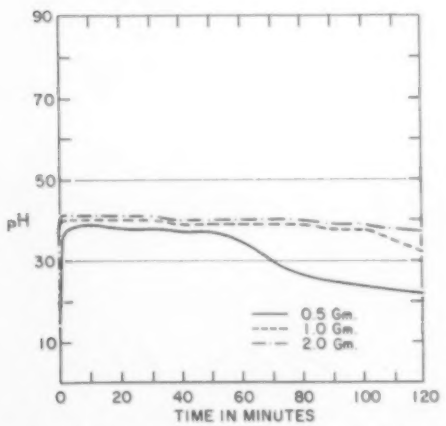

Fig. 5.—Aluminum hydroxide-glycine dried gel type F-4B. Procedure of Holbert, Noble, and Grote modified.

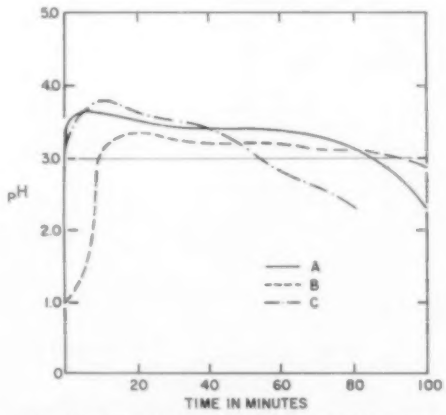

Fig. 6.—Antacid activity of three aluminum antacids—1.0 Gm. basis. *A*, aluminum hydroxide-glycine dried gel type F-4B. *B*, aluminum hydroxide dried gel U. S. P. *C*, aluminum dihydroxy aminoacetate. Method of Fuchs.

shown are values obtained on 1-Gm. samples of AHG dried gels, aluminum hydroxide dried gel U. S. P., and aluminum dihydroxy aminoacetate. The data for the three dried aluminum antacids is plotted in Fig. 6.

Effect of Age.—To show that aging at ambient temperatures has little or no effect on the antacid properties of aluminum hydroxide-glycine dried gel Type F-4B, a seven-year old sample was evaluated by the Holbert, Noble, and Grote method. The results are plotted in Fig. 7 on the basis of 0.5, 1.0, and 2.0 Gm.

Dry Blends.—In contrast with the very rapid and prolonged antacid action of aluminum hydroxide-glycine dried gels in the optimum pH range of 3 to 5, dry blends of aluminum hydroxide dried gel U. S. P. and glycine are somewhat less reactive than aluminum hydroxide dried gel by itself. This may be

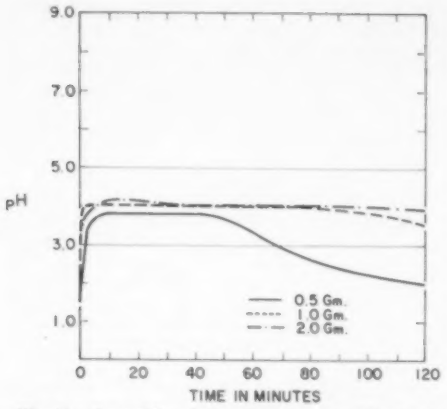

Fig. 7.—Antacid activity of seven year-old sample of aluminum hydroxide-glycine dried gel type F-4B. Procedure of Holbert, Noble, and Grote modified.

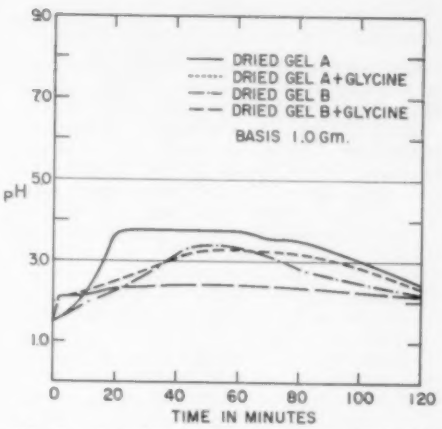

Fig. 8.—Antacid activity of aluminum hydroxide dried gel U. S. P. compared with dry blends with glycine, 1 mole aluminum oxide to 1 mole glycine. Procedure of Holbert, Noble, and Grote modified.

TABLE V.—EFFECT OF GLYCINE ON REACTION VELOCITY OF ALUMINUM HYDROXIDE DRIED GEL U. S. P. WITH 0.1 *N* HYDROCHLORIC ACID[a]

	Reheis Method Time, sec.	Mutch Method Time, sec.
Aluminum hydroxide dried gel U. S. P. (sample A)	230	410
Aluminum hydroxide dried gel U. S. P. (sample A) + glycine, dry blend	365	580
Aluminum hydroxide dried gel U. S. P. (sample B)	375	...
Aluminum hydroxide dried gel U. S. P. (sample B) + glycine, dry blend	655	...

[a] One mole of glycine per mole of aluminum oxide.

seen in Table V which shows Reheis and Mutch reaction velocities on two samples of aluminum hydroxide dried gels U. S. P. and the same samples blended with glycine in the ratio of 1 mole of aluminum oxide to 1 mole of glycine. *In vitro* antacid evaluation data by the Holbert, Noble, and Grote procedure are shown for two different samples of dried aluminum hydroxide gel and blends of the same dried gels with glycine in the ratio of 1 mole aluminum oxide to 1 mole of glycine in Fig. 8. On the basis of these data one may conclude that glycine has an adverse effect on the antacid properties of aluminum hydroxide dried gel.

Aluminum-Magnesium Hydroxide-Glycine Dried Gel.—Liquid reactive aluminum hydroxide gel, properly combined with magnesium hydroxide, forms the basis of some of the most widely and successfully used preparations for the medical management of peptic ulcer (16, 17).

In these preparations, the buffering action of the aluminum ion is so great that equilibrium pH values with gastric acid are in the range of 3.8 to 4.5, in spite of the highly basic magnesium hydroxide. Dry blends of magnesium hydroxide with aluminum hydroxide dried gel show a high initial surge to pH 6.5 or higher and are not as prolonged in antacid

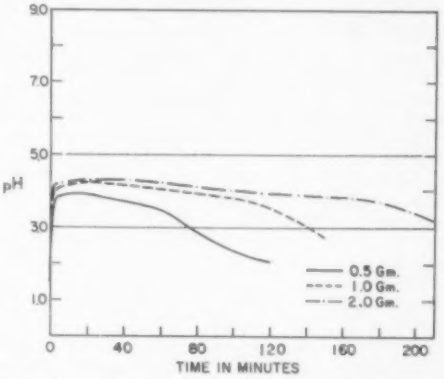

Fig. 9.—Antacid activity of aluminum-magnesium hydroxide-glycine dried gel type F-4BM. Mole ratio: alumina : magnesium hydroxide : glycine 1 : 0.83 : 0.68. Procedure of Holbert, Noble, and Grote modified.

activity as the liquid preparations. However, when finely divided magnesium hydroxide is added to the aluminum hydroxide-glycine gel before drying, the resulting dried gel exhibits prompt and very prolonged antacid activity in the pH range of 3.0 to 4.5, which is comparable to liquid products. This is demonstrated in Fig. 9 by the Holbert, Noble, and Grote method.

In Fig. 9, the antacid activity of a sample of an aluminum-magnesium hydroxide-glycine (AMHG) dried gel type F-4BM is plotted for 0.5, 1.0, and 2.0-Gm. dose levels. AMHG dried gel type F-4BM contains 0.83 mole magnesium hydroxide, 0.68 mole glycine, and 1 mole aluminum hydroxide (as Al_2O_3).

Each gram of a typical F-4BM powder contains approximately 664 mg. aluminum hydroxide (calculated as 50% aluminum oxide), 159 mg. magnesium hydroxide, and 166 mg. glycine. F-4BM also exhibits a pleasant bland, slightly sweet taste property as does F-4B.

RESULTS

Reaction Rate with Gastric Acid.—The reaction velocity data in Table I shows that the two AHG gels are slightly slower in reaction with 0.1 *N* hydrochloric acid than a highly reactive aluminum hydroxide gel. The difference is probably not sufficient to have a significant physiological effect. AHG gels, as well as aluminum hydroxide gels, are completely reactive with gastric acid and neutralize 0.1 *N* hydrochloric acid in theoretical amounts (586 ml. per Gm. aluminum oxide).

Table II reveals that the reaction velocities of AHG dried gels and ADA are more rapid than aluminum hydroxide dried gel. Both AHG dried gels react at a somewhat higher rate than ADA. The acid consuming capacity per gram of aluminum oxide shows that all four samples neutralize 94 to 98% of the theoretical amount of 0.1 *N* hydrochloric acid. Since the acid consuming capacity per gram of alumina is approximately the same for all four antacids, our data does not support the conclusions of Krantz, *et al.* (1), that "on the basis of the aluminum content, basic aluminum aminoacetate is 42% more efficient in acid consuming power than dried aluminum hydroxide gel." This conclusion and its implications has received universal recognition in the pharmaceutical and medical literature including the publications of other investigators (2, 5, 7). The presumption that the amino group in ADA provides extra buffering capacity is without experimental evidence. It also follows that for equal volumes of gastric juice to be neutralized to pH 3.5 to 4, equal amounts of aluminum chloride are formed for either antacid. According to the data of Sorensen (18), at pH 4 glycine can bind less than 0.05 equivalents of hydrogen ions per mole of glycine, which is an insignificant amount.

***In Vitro* Antacid Activity; Holbert, Noble, and Grote Procedure Modified.**—We consider that our modification of the Holbert, Noble, and Grote procedure for evaluation of antacids to be as stringent a test as it is possible to devise. It is made such by the dynamic conditions of constant influx of fresh artificial gastric juice and steady outflow of antacid-gastric juice mixture. The data by this method, which are shown in Table III and plotted in Figs. 1–5, indicate the following: (*a*) The antacid activity of AHG gels is comparable to highly reactive aluminum hydroxide gel. (*b*) Small volume doses of AHG gels are very effective; for example, a 5-ml. dose of 8.0% aluminum oxide F-4B gel is equivalent to 10 ml. of aluminum hydroxide gel U. S. P. (*c*) AHG dried gels, types F-4A and F-4B, are rapid and prolonged in antacid activity in the pH range of 3 to 4.5. (*d*) Type F-4B with only one-half the weight of glycine per weight of aluminum is faster and more prolonged than either F-4A or ADA. (*e*) When compared on a 1-Gm. basis, dried aluminum hydroxide gel is much slower and less prolonged than AHG dried gels or ADA. (*f*) The antacid activity of a 1-Gm. dose of aluminum hydroxide-glycine gel, type F-4B is comparable to a 10-ml. dose of a highly reactive aluminum hydroxide gel U. S. P. of clinically proved effectiveness. (*g*) The antacid activity of AHG dried gel, type F-4A and ADA are similar.

***In Vitro* Antacid Activity, Method of Fuchs.**—The antacid evaluation data by this method, which are seen in Table IV and Fig. 6, reveal the following information: (*a*) The antacid activity of AHG gels and aluminum hydroxide gel is comparable. (*b*) The antacid activity of AHG dried gels, types F-4A and F-4B is somewhat greater than ADA. (*c*) Aluminum hydroxide dried gel is slower but more prolonged in activity than AHG dried gels and ADA.

DISCUSSION

The requirements of a successful modern antacid have been well stated by several investigators (15, 19, 20) and need not be repeated here. By these standards, reactive liquid aluminum hydroxide gels alone or effectively combined with magnesium hydroxide or magnesium trisilicate come close to the ideal. They have been proved to be clinically effective.

Our data has shown that the addition of only one mole of glycine to one mole of highly reactive aluminum hydroxide gel (as aluminum oxide) with or without a balanced amount of magnesium hydroxide gel before drying, stabilizes the highly complex structure during and after the desiccation process. The result is a stable dried aluminum hydroxide gel or aluminum-magnesium hydroxide gel that has antacid properties similar to those of clinically effective liquid gels. The bland, pleasant tasting powders form tablets which not only exhibit the outstanding antacid properties of liquid gels but are also smooth, nonchalky, and well tolerated.

The prompt and prolonged antacid activity of aluminum hydroxide-glycine dried gel, type F-4B has also been substantiated in the clinic.

SUMMARY

1. The preparation and properties of two new combinations of aluminum hydroxide and glycine in both gel and dried gel form have been described.

2. Evaluation of the antacid properties of the AHG gels by five *in vitro* methods demonstrates that they are both prompt and prolonged in buffering gastric acid in the optimum pH range of 3 to 5. The reaction rate is slightly less than aluminum hydroxide gels. They are especially suitable for liquid preparations of very high acid neutralizing power and for gels in intragastric therapy of peptic ulcer.

3. *In vitro* evaluation of the antacid properties of aluminum hydroxide-glycine dried gels by five methods shows that they compare favorably with clinically effective liquid aluminum hydroxide gel in speed and duration of activity in the optimum pH range of 3 to 5.

4. AHG dried gel, type F-4B, having one mole each of alumina and glycine, is more prompt and prolonged in antacid activity than type F-4A or aluminum dihydroxy aminoacetate. It is also more inexpensive to manufacture and hence, is more economical to use. Its outstanding antacid properties are unaffected by time.

5. Tablets made from F-4B dried gel, in addition to being prompt and prolonged in buffering power, have a smooth, nonchalky, pleasant taste property.

6. A modified F-4B dried gel, having a balanced weight ratio of aluminum hydroxide-magnesium hydroxide-glycine of 4:1:1, has been shown to have prompt and prolonged antacid action similar to that of clinically effective liquid aluminum-magnesium hydroxide gels.

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Preparation and Properties of New Gastric Antacids IV

Aluminum Hydroxide-Magnesium Trisilicate

By STEWART M. BEEKMAN

The preparation and properties of several new combinations of aluminum hydroxide and magnesium trisilicate (AHMT) in gel and dried gel form are described. The mole ratios of alumina to magnesia of the various products range from 8–2 to 0.5–2. The antacid properties were evaluated by a very stringent *in vitro* method—our modification of the procedure of Holbert, Noble, and Grote. The antacid properties of the five gels and dried gels are shown to be prompt and prolonged in the optimum pH range of 3 to 5. The excellent antacid properties of the AHMT dried gels prepared by our process are contrasted with the results exhibited by usual dry blends of aluminum hydroxide dried gel with magnesium trisilicate powder.

THE THREE preceding papers (1–3) in this series of *in vitro* studies described mixtures of aluminum hydroxide with (*a*) magnesium carbonate, (*b*) various proteins and protein products including nonfat milk, and (*c*) glycine. All were new combinations of old antacids by new processes. They were shown to be prompt and prolonged in buffering gastric juice in the optimum pH range of 3 to 5. This paper is a continuation of these studies and is concerned with new combinations of aluminum hydroxide with magnesium trisilicate in gel and dried gel form.

Roch's (4) first use of a dry colloidal aluminum hydroxide in 1922 was followed twelve years later by Einsel, Adams, and Myers (5) who introduced a gelatinous reactive aluminum hydroxide gel into therapeutics as a gastric antacid. In 1936, Mutch (6–9) presented his well-known papers on the antacid use of synthetic hydrated magnesium trisilicate, having the formula $2MgO \cdot 3SiO_2 nH_2O$. Both materials received a great deal of attention from gastroenterologists; they represented a new concept in antacid therapy. Each was held by some investigators to be the ideal antacid. It was found that combinations of the two complemented each other in some properties. Liquid suspensions containing both materials have given excellent clinical results in antacid therapy of peptic ulcer. Tabletted products have not been as successful as the liquid suspensions. It was our purpose to develop improved aluminum hydroxide-magnesium trisilicate blends in both gel and dried gel form.

The aluminum hydroxide-magnesium trisilicate (AHMT) gels described in this paper can be made by several processes with varying results. The

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TABLE I.—COMPOSITION OF ALUMINUM HYDROXIDE-MAGNESIUM TRISILICATE COMPRESSED GELS

Type, Designation	Mole Ratio Al_2O_3:MgO:SiO_2	Al_2O_3, %	MgO, %	SiO_2, %	Aluminum Hydroxide, %[a]	Magnesium Trisilicate, %[b]	Acid Consuming Capacity[c]
F-MAS 823	8:2:3	8.2	0.8	1.8	16.1	3.7	50
F-MAS 423	4:2:3	6.9	1.4	3.1	13.5	6.2	46
F-MAS 223	2:2:3	5.3	2.1	4.6	10.4	9.5	42
F-MAS 123	1:2:3	3.6	2.9	6.4	7.0	13.0	35
F-MAS 146	0.5:2:3	2.2	3.5	7.7	4.3	15.9	29

[a] Calculated as 51% aluminum oxide. [b] Calculated as 22% magnesium oxide. [c] ml. 0.1 *N* hydrochloric acid per Gm.

TABLE II.—TYPICAL ANALYSES OF ALUMINUM HYDROXIDE-MAGNESIUM TRISILICATE DRIED GELS

Type, Designation	Al_2O_3, %	MgO, %	SiO_2, %	Relative Amount per Gm. Aluminum Hydroxide, mg.[a]	Relative Amount per Gm. Magnesium Trisilicate, mg.[b]	Approx. Wt. Ratio Aluminum Hydroxide to Magnesium Trisilicate	Acid Consuming Capacity[c]
F-MAS 823	43.2	4.7	10.3	847	213	4:1	270
F-MAS 423	36.9	7.9	17.4	722	359	2:1	246
F-MAS 223	28.1	11.9	26.4	550	540	1:1	210
F-MAS 123	19.0	17.0	36.1	372	773	1:2	178
F-MAS 146	11.2	19.5	43.0	220	886	1:4	148

[a] Calculated as 51% aluminum oxide. [b] Calculated as 22% magnesium oxide. [c] ml. 0.1 *N* hydrochloric acid per Gm.

first method is to precipitate separately a highly reactive aluminum hydroxide gel and magnesium trisilicate gel, combine the two slurries, and filter and wash the combined separately precipitated gels until free of soluble salts. The washed filter cake may be milled to provide a smooth, homogenous mixed hydrogel. Table I shows the designation and composition of a typical series of five AHMT gels, having aluminum oxide to magnesium oxide mole ratios of from 8:2 to 0.5:2. These gels when properly diluted with water are thixotropic fluid suspensions which react readily with gastric acid. Less viscous gels may be made by the preparation of a finely divided, highly reactive magnesium trisilicate powder and thoroughly dispersing it in a highly reactive aluminum hydroxide gel.

The AHMT dried gels are prepared by the careful desiccation of intimate blends of a highly reactive aluminum hydroxide gel with a low temperature magnesium trisilicate gel. Drying is best carried out by spray drying or by low temperature air drying followed by milling to fine particle size. The dried gels are fine, bland, tasteless, odorless, smooth, white powders. They react rapidly with 0.1 *N* hydrochloric acid. Table II shows analyses of typical samples of various AHMT dried gels.

EXPERIMENTAL

Aluminum Hydroxide-Magnesium Trisilicate Gels.—Fluid suspensions were prepared from each of the five types of AHMT gels on the basis of 4.0% aluminum oxide for F-MAS 823, F-MAS 423, and F-MAS 223; 3.0% aluminum oxide for F-MAS 123; and 2.0% aluminum oxide for F-MAS 146. The suspensions were made with distilled water, using a laboratory dispersator. The acid consuming capacity of each gel was determined. The values which are shown in Table I in terms of 1-Gm. samples of the undiluted gels approach the theoretical for the amount of alumina and magnesia present.

Samples of the fluid suspensions containing 0.42 Gm. aluminum oxide (10 ml. for 4% aluminum oxide gels, 13.3 ml. for 3% aluminum oxide gels, and 20 ml. for 2% aluminum oxide gels) were evaluated by our modification of the procedure of Holbert, Noble, and Grote. Details of the acid consuming capacity test and the Holbert, Noble, and Grote method may be found in a previous paper (1). The data obtained are plotted in Fig. 1.

Aluminum Hydroxide-Magnesium Trisilicate Dried Gels.—A series of five AHMT dried gels was prepared, having analyses similar to those shown in

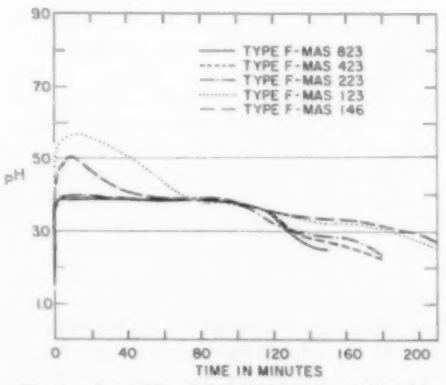

Fig. 1.—Antacid activity of aluminum hydroxide-magnesium trisilicate gels. Basis: 0.42 Gm. aluminum oxide. Procedure of Holbert, Noble, and Grote modified.

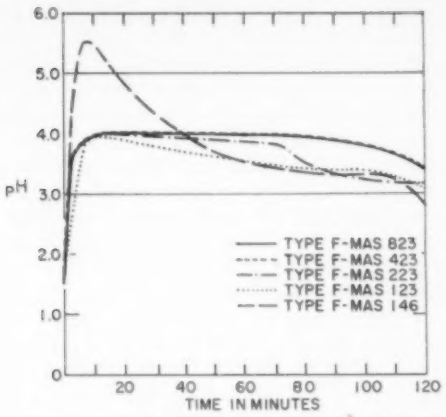

Fig. 2.—Antacid activity of aluminum hydroxide-magnesium trisilicate dried gels. Basis: 1.0 Gm. Procedure of Holbert, Noble, and Grote modified.

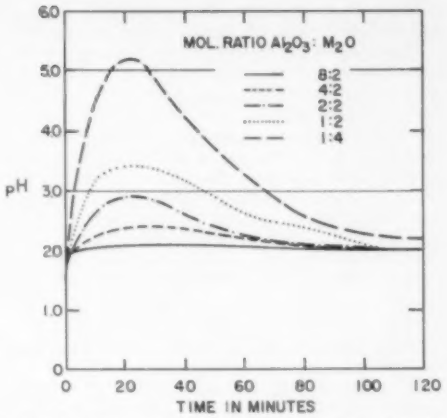

Fig. 3.—Antacid activity of aluminum hydroxide dried gel U. S. P.-magnesium trisilicate U. S. P. dry blends. Basis: 1.0 Gm. Procedure of Holbert, Noble, and Grote modified.

Table II. The acid consuming capacity was determined and the antacid characteristics measured by the modified Holbert, Noble, and Grote method. The data are shown in Fig. 2.

Dry Blends.—A series of five dry blends was prepared from samples of aluminum hydroxide dried gel U. S. P. and magnesium trisilicate U. S. P. The dry blends were made on the same mole ratio basis of aluminum oxide to magnesium oxide as the co-dried gels described above. Each of these blends was evaluated for antacid activity by the modified *in vitro* method of Holbert, Noble, and Grote. Values obtained are shown in Fig. 3.

Magnesium Trisilicate.—To determine any possible variation in antacid activity, ten different samples of magnesium trisilicate U. S. P. were evaluated by our modified Holbert, Noble, and Grote method. All samples are commercial products from various manufacturers except F and G, which are experimental preparations prepared in our pilot plant. The latter sample is an undried magnesium trisilicate gel (antacid evaluation on a dry basis). Figure 4 shows the wide variation in antacid properties in products which meet U. S. P. specifications.

Aluminum Hydroxide Dried Gel.—Three different samples of aluminum hydroxide dried gel were also evaluated by the Holbert, Noble, and Grote method, and the results are shown in Fig. 5.

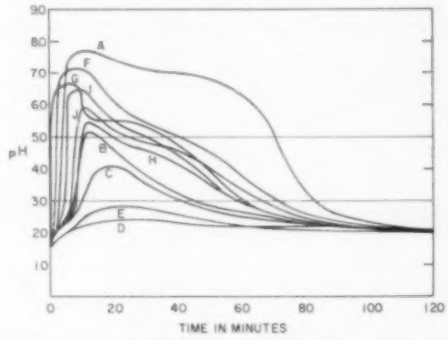

Fig. 4.—Antacid activity of various samples of magnesium trisilicate U. S. P. 1.0-Gm. basis. Procedure of Holbert, Noble, and Grote modified.

RESULTS

Undried Gels.—The results of our very stringent *in vitro* method of antacid evaluation show that all five AHMT gels are very prompt in raising the pH of gastric juice to the optimum pH level of 3 to 5 and prolonged in maintaining the level in this range. Only in the case of two samples, F-MAS 123 and F-MAS 146, did the pH rise slightly above 5. Basically, the curves show the overall antacid effect of adding increasing amounts of a highly reactive magnesium trisilicate gel to a very reactive aluminum hydroxide gel. The antacid activity of the magnesium trisilicate gel used is shown in curve G, Fig. 4.

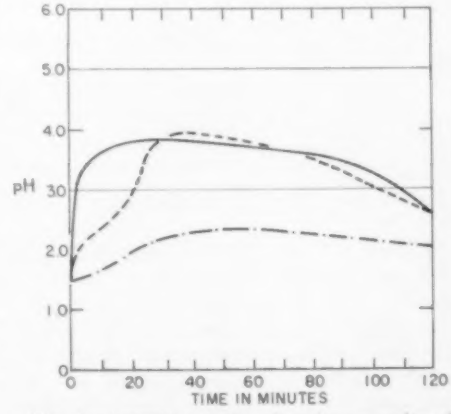

Fig. 5.—Antacid activity of various samples of aluminum hydroxide dried gel. U. S. P. 1.0-Gm. basis. Procedure of Holbert, Noble, and Grote modified.

Dried Gels.—*In vitro* antacid evaluation data on the various samples of AHMT dried gels, based on 1-Gm. samples, show prompt and prolonged buffering action in the pH range of 3 to 5. All products maintained the pH level above pH 3 for about two hours. These samples vary widely in the ratio of the two components present, from four parts of aluminum hydroxide per part of magnesium trisilicate to the reverse ratio. As might be expected from the acid consuming capacities of the two components and the various co-dried mixtures, the antacid activity of samples containing the greatest amount of magnesium trisilicate were the most prompt. Those with the largest amount of aluminum hydroxide were most prolonged. A good compromise from the viewpoint of antacid activity is type F-MAS 223, which contains equal parts of the two antacids.

Dry Blends.—By contrast with the excellent antacid properties shown by 1.0-Gm. samples of aluminum hydroxide-magnesium trisilicate co-dried gels, the data which are plotted in Fig. 3 shows the relatively poor antacid properties of dry blends of the two components.

Adsorption Characteristics.—Co-dried gels of aluminum hydroxide-magnesium trisilicate, in addition to their excellent antacid properties, also exhibit excellent adsorption characteristics toward many positive and negative dyes. It is possible that adsorption agents which adsorb over a wide range may be prepared by this method.

SUMMARY

1. The preparation and properties of five aluminum hydroxide-magnesium trisilicate gels and dried gels having a wide variation of the two components have been described.

2. The antacid properties of aqueous suspensions of the five gels by a very stringent *in vitro* technique were found to be outstanding in the optimum pH range of 3 to 5.

3. The antacid properties of the five co-dried gels were compared with dry blends of aluminum hydroxide dried gel U. S. P. and magnesium trisilicate U. S. P. The former were prompt and prolonged in the optimum pH range. The dry blends were found to be very poor.

4. The antacid activity of the various co-dried AHMT gels compares favorably with clinically effective liquid suspensions of aluminum hydroxide and magnesium trisilicate.

5. AHMT dried gel systems may be altered by the inclusion of other modifying agents such as glycine or sorbitol.

6. AHMT dried gels properly stored for more than a year show only a slight diminution in antacid activity.

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Effect of Acorus Oil *in Vitro* on the Respiration of Rat Brain

By N. S. DHALLA, C. L. MALHOTRA, and M. S. SASTRY

By Warburg manometric technique, the steam volatile oil of *Acorus calamus* L. was found to inhibit the oxygen uptake of rat brain homogenate in phosphate buffer. It produced a mild, moderate, and marked inhibition with sodium succinate, sodium glutamate, and glucose, respectively, as respiratory substrates. Lysergic acid diethylamide exhibited very little inhibition on the endogenous respiration and also partially reduced the inhibition caused by acorus oil. In combination with 5-hydroxytryptamine, the inhibitory action of the drug was considerably potentiated.

ACORUS CALAMUS L., is a popular drug in the Ayurvedic system of medicine in India and is widely used for the treatment of various nervous disorders (1). Agarwal, *et al.* (2), in 1956 reported that the total extract of the rhizomes of the plant possessed sedative and analgesic properties. The recent detailed pharmacological investigations carried out by Dandiya, *et al.* (3, 4), showed that the activity of the drug was due to the steam volatile oil. Their main findings are that the oil potentiated the hypnotic activity of barbiturates and ethanol, exacerbated the tonic seizures provoked by convulsive doses of metrazol, reduced the amphetamine toxicity, and caused hypothermia.

It is known that certain drugs produce effects on the oxidative process of the brain *in vitro* and

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that the *in vivo* mechanism can partly be explained in terms of the effects on the enzymatic process (5). Since Dandiya, *et al.*, have suggested that the acorus oil acts similarly to reserpine and also that the mechanism of action is common to that of some ataractic agents, it was considered of great interest to evaluate the mode of action of acorus oil *in vitro* using various substrates and other drugs in combination.

MATERIALS AND METHODS

Albino rats weighing 100–150 Gm. were decapitated and the brain was immediately removed, weighed, washed, and transferred to a previously chilled glass tissue grinder containing Krebs-Ringer phosphate buffer, pH 7.3 (6). The brain was homogenized and the homogenate was diluted with phosphate buffer so that each ml. of the homogenate contained 100 mg. of the fresh tissue.

Oxygen uptake was measured at ten-minute intervals by the Warburg manometric method. The gas phase was air and the temperature of the bath was 38°. Each reaction vessel contained 1 ml. of the homogenate and the solvent control (0.3 ml.) with or without the drug. In those experiments where substrates were used, 0.3 ml. of glucose (0.33 *M*), or 0.3 ml. of sodium glutamate (2.0 *M*), or 0.3 ml. of sodium succinate (0.33 *M*), all dissolved in phosphate buffer, was added. In some experiments 0.3 ml. of 10^{-3} *M* lysergic acid diethylamide (LSD-25) or 0.3 ml. of 10^{-2} *M* 5-hydroxytryptamine (5-HT), in phosphate buffer, was added. The total volume of the fluid in the flask was always made up to 3 ml. with phosphate buffer. The central well of the flask contained 0.2 ml. of 20% potassium hydroxide absorbed on filter paper. The drug with or without substrates was tipped in from the side arm at zero time after ten minutes of equilibration. All the measurements were performed in triplicate and the rate of oxygen uptake, Q_{O_2}, for the first sixty minutes was calculated as μL. of oxygen per hour per 100 mg. of the brain tissue.

The acorus oil was suspended in phosphate buffer containing 1% Tween 80 which also served as control throughout the investigation. The effect of solvent control was found to be insignificant and hence omitted from the text. All the concentrations of drug refer to the final concentration in the reaction vessel.

RESULTS AND DISCUSSION

The effect of various concentrations of acorus oil on the respiration of rat brain homogenate in phosphate buffer is given in Table I. Although the brain homogenate used for each concentration in different experiments varied, the comparison of effect was made on the same batch. Hence, the percentage effect remains within the experimental limits. Acorus oil caused varying degrees of respiratory inhibition with change in concentration: 20% effect was noted with 1:16,000, whereas in concentration of 1:500, it exhibited 70% inhibition.

The ED_{50} (with 19/20 confidence limits) calculated by the method of Litchfield and Wilcoxon (7) was found to be 1:1,980 (1:1,309–1:2,594). This optimum concentration (1:2,000) was selected for studying the effect on respiration in the presence of various substrates and in combination with LSD-25 and 5-HT to find out the influence exerted by them on its activity.

TABLE I.—EFFECT OF VARIOUS CONCENTRATIONS OF ACORUS OIL ON THE OXYGEN UPTAKE OF RAT BRAIN HOMOGENATE IN PHOSPHATE BUFFER

Concentration of Drug	Q_{O_2}[a] without Drug	Q_{O_2}[a] with Drug	Inhibition, %
1:16,000 (3)[b]	69.7 ± 2.5	56.0 ± 2.0	19.7 ± 0.7
1:8,000 (5)	69.7 ± 1.5	47.3 ± 1.7	29.3 ± 1.0
1:4,000 (4)	69.7 ± 2.9	41.8 ± 1.0	41.0 ± 1.2
1:2,000 (6)	70.9 ± 3.1	26.8 ± 1.8	47.7 ± 2.7
1:1,000 (4)	62.2 ± 2.8	25.5 ± 0.7	57.0 ± 2.4
1:500 (3)	52.3 ± 1.0	15.3 ± 0.3	71.3 ± 0.8

[a] Calculated as μL./hr./100 mg. of brain tissue.
[b] Figures within parentheses show the number of experiments in each study.

The rates of oxygen uptake at different intervals by 100 mg. of the rat brain homogenate in the presence of glucose (0.033 *M*), sodium glutamate (0.2 *M*), and sodium succinate (0.033 *M*), with and without acorus oil, are plotted in Fig. 1. Each reading is the average of 12 to 21 reaction vessels in several experiments and is within the variable range of ±5.

It was found that the percentage inhibition in glucose, sodium glutamate, and sodium succinate was 43.1 ± 2.9, 34.1 ± 1.5, and 6.7 ± 1.5, respectively. The inhibition was maximum in glucose and almost negligible in sodium succinate. It is suggested that the action of acorus oil might be localized in the citric acid cycle.

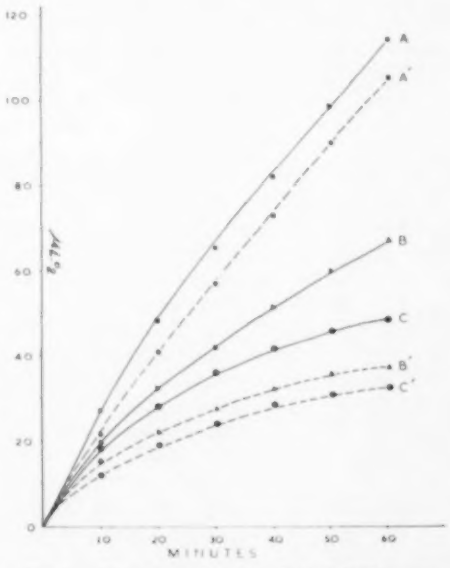

Fig. 1.—The effect of acorus oil (1:2,000) on oxygen uptake of rat brain homogenate in various substrates. *A*, Sodium succinate (0.033 *M*); *A'* sodium succinate + acorus oil; *B*, glucose (0.033 *M*); *B'*, glucose + acorus oil; *C*, sodium glutamate (0.2 *M*); *C'*, sodium glutamate + acorus oil.

TABLE II.—EFFECT OF LYSERGIC ACID DIETHYLAMIDE AND 5-HYDROXYTRYPTAMINE WITH OR WITHOUT ACORUS OIL ON THE RESPIRATION OF RAT BRAIN

Drug	Q_{O_2} in μL./hr./100 mg. of Brain Tissue	Inhibition, %	
Control	60.3 ± 2.6 (7)[a]	...	
Acorus oil (2×10^{-3})	32.3 ± 2.0 $P < 0.001$[b] (7)	46.5 ± 0.8	
LSD-25 (10^{-4} *M*)	53.5 ± 2.8 $P > 0.05$ (3)	10.7 ± 0.5	
LSD-25 (10^{-4} *M*) plus acorus oil (2×10^{-3})	42.5 ± 1.2 $P < 0.001$ (3)	37.3 ± 1.3	$P_t > 0.02$[c]
5-HT (10^{-3} *M*)	40.2 ± 0.3 $P < 0.001$ (4)	27.3 ± 0.8	
5-HT (10^{-3} *M*) plus acorus oil (2×10^{-3})	18.3 ± 2.7 $P < 0.001$ (4)	67.2 ± 2.5	$P_t < 0.001$

[a] Figures within parentheses show the number of experiments in each study.
[b] Probability (*P*) calculated by *t* test.
[c] Probability (P_t) calculated by *t* test due to the influence of LSD-25 and 5-HT on acorus oil.

The influence of LSD-25 (10^{-4} *M*) and 5-HT (10^{-3} *M*) on the respiration, with or without acorus oil (1:2,000), has been recorded in Table II. It will be seen from this Table that the inhibition produced by LSD-25 is of very little significance. It was, however, interesting to note that the addition of LSD-25 to the Warburg vessel containing acorus oil slightly reduced the inhibitive action of the oil; 5-HT, on the other hand, potentiated the action of oil considerably, even though it had individually inhibitory action on the respiration of brain.

It has been postulated that mental aberration induced by LSD-25 might be due to a metabolic antagonism between LSD-25 and 5-HT (8). Starbuck and Heim (9) have reported that LSD-25 has no effect on chlorpromazine while 5-HT partially inhibited the action of chlorpromazine *in vitro*. From our observations it is evident that LSD-25 partially reduced the inhibition caused by acorus oil and is in agreement with *in vivo* results reported by Dandiya, *et al.* (4). Potentiation caused by 5-HT *in vitro* on the inhibiting action of acorus oil lends further support to the suggested mechanism *in vivo*.

SUMMARY

1. The effect on the rate of respiration of the rat brain homogenate with various concentrations of the steam volatile oil of *Acorus calamus* L. has been studied by manometric technique.

2. Acorus oil was found to inhibit the oxygen uptake of brain tissue to a varying degree and ED_{50} was 1:1,980.

3. In the presence of glucose, sodium glutamate, and sodium succinate, acorus oil exhibited 43.1, 34.1, and 6.7% inhibition, respectively.

4. LSD-25 reduced the inhibitory action of acorus oil while there was potentiation with 5-HT.

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Perchloric Acid Titrations with a Commercially Available Electrode

By JOHN J. HEFFERREN and HENRY M. KOEHLER

The combination electrode[1] is a suitable electrode for potentiometric titrations in water miscible and immiscible, nonaqueous solvents with perchloric acid in glacial acetic acid or dioxane as a titrant. The construction of the electrode is such that high resistance solvents and mercuric acetate contamination should not limit its usefulness. When prolonged exposure of the electrode to glacial acetic acid is anticipated, an alternate electrolyte should be considered.

Nonaqueous methods utilizing perchloric acid as a titrant have found wide acceptance in the analysis of amines and amine salts which constitute a very high percentage of medicinal agents in use today. While there are many indicators which can be used in these titrations, potentiometric titrations are necessary to establish the appropriate indicator color change at the equivalence point (Fig. 1) and to determine quantitatively the titratable compound in highly colored media.

The electrode requirements are an important factor in the success of a potentiometric nonaqueous titration because of the nature of solvents and titrants which are used. Some of the useful electrode characteristics for nonaqueous titrations are: good proximity of the two electrodes, stability at the end point, resistance to contamination by the titration medium, and compatibility with the titration reactants.

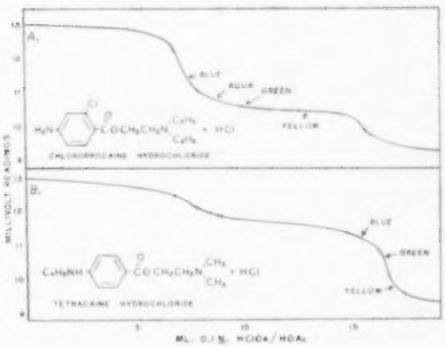

Fig. 1.—Potentiometric titration of chloroprocaine and tetracaine hydrochlorides in glacial acetic acid with perchloric acid in glacial acetic acid. Mercuric acetate in glacial acetic acid and crystal violet indicator were added.

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Samples of the anesthetics were generously supplied by their manufacturers: chloroprocaine hydrochloride by Lederle Laboratories Division, American Cyanamid Co., lidocaine by Astra Pharmaceutical Products, Inc., and tetracaine hydrochloride by Cooke-Waite Laboratories, Inc., subsidiary of Sterling Drug Inc.

[1] The combination electrode No. 39142 now being manufactured has an improved design stopper which is made of a more solvent-resistant rubber. This electrode should be less likely to cause electrolyte contamination of the nonaqueous titration medium; however, an electrolyte containing a minimal amount of chloride should be used.

Pifer and co-workers (1) utilized a combination of the usual microcalomel and glass electrodes in a specially prepared titration flask. The calomel electrode was mounted in the side of the flask, thereby placing the tips of the electrodes very close to each other. Such an arrangement made it possible to titrate effectively in high resistance solvents such as chloroform.

The calomel electrode in this system, like any other calomel electrode, is poisoned by the mercuric acetate which is used frequently in nonaqueous perchloric acid titrations to tie up the ionizable halogen such as those of amine hydrochlorides. While this contamination can largely be avoided by frequent changing of the electrolyte in the calomel electrode, once contamination has occurred there is a fluctuation of the potential at the end point (2). The use of a glass electrode paired with a silver wire coated with silver chloride (3) or a commercial silver chloride electrode circumvented this problem of mercuric acetate contamination; however, these electrode combinations were also reported to be somewhat unstable at the end point (4). Glenn (4) prepared an isolation cell for a commercial silver-silver chloride reference electrode to achieve greater end point stability. The isolation cell contained a saturated solution of potassium chloride in glacial acetic acid which flowed through a ground glass joint to create a junction between the titration medium and the silver-silver chloride electrode.

EXPERIMENTAL

Apparatus.—A Beckman Zeromatic pH meter equipped with the Beckman combination electrode, No. 39142.

Reagents.—All solvents and reagents used were analytical reagent grade except acetonitrile which was practical grade.

The dioxane used in the preparation of the 0.1 *N* perchloric acid in dioxane reagent was redistilled over sodium ribbon. The acetonitrile was mechanically shaken with Amberlite IRC-50 ion-exchange resin and stored over this same resin.

The indicators used were a 0.1% solution of crystal violet in glacial acetic acid and a 0.2% solution of methyl red in methanol.

Alternate electrolyte was 10% potassium nitrate equilibrated with silver chloride.

Procedure.—(*A*) A 1 to 1.5-meq. quantity of drug or potassium acid phthalate was dissolved in 50 ml. of glacial acetic acid, using heat if necessary. The solution was cooled to room temperature, indicator added, and the titration with 0.1 *N* perchloric acid in glacial acetic acid was followed potentiometrically and colorimetrically.

(*B*) With compounds containing ionizable halogen, 10 ml. of 6% mercuric acetate in glacial acetic acid were added to the titration mixture.

(*C*) With solvents other than glacial acetic acid, 0.1 *N* perchloric acid in dioxane was used as a titrant and methyl red was used as an indicator.

COMMENTS

When the ordinary calomel-glass electrode combination did not work well for nonaqueous titration with the pH meter-potentiometer available in our laboratory, a commercial combination electrode which appeared to have many of the desired characteristics for nonaqueous titrations was tried. The combination electrode (Fig. 2) consisted of an ordinary glass electrode in the center surrounded by a second electrode consisting of silver wire with silver chloride-coated tip and potassium chloride-silver chloride electrolyte. The electrolyte suggested by the firm is a 4 *M* solution of potassium chloride which has been equilibrated with silver chloride. The electrolyte maintains contact between this outer half cell of the electrode and the titration mixture by diffusion through a linen thread wrapped around a silicone rubber stopper at the bottom of the electrode.

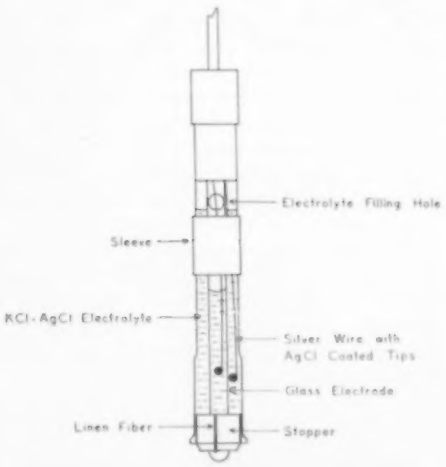

Fig. 2.—Commercially available combination electrode.

Titrations in the Usual Nonaqueous Solvents.—In Fig. 1, typical potentiometric titration curves together with the accompanying crystal violet indicator changes are illustrated for two amine hydrochlorides. In the titration of such compounds with a strongly basic aliphatic tertiary amine and a less basic aromatic primary or secondary amine, a much better titration curve is obtained by heating the titration mixture with acetic anhydride. The acetic anhydride effectively ties up the primary and secondary amines as the corresponding acetates, thus leaving the strongly basic tertiary amine to be titrated.

When chloroform was used as a titration solvent, more stable readings were obtained by adding sufficient acetonitrile to give a chloroform-acetonitrile ratio of 4 to 1. Figure 3 is an example of the potentiometric titration curve of the free base of lidocaine hydrochloride. During titrations in such water-immiscible solvents, a crust of solute from the electrolyte may form occasionally at the tip of the fiber in contact with the titration medium This crust formation has not caused any difficulty or interference.

Continuous Use of Combination Electrode in Nonaqueous Solvents.—When a large series of titrations using glacial acetic acid as a titration medium was being run each day over a period of a few weeks, high results were recorded in the later stages of the work. This was particularly noticeable for those compounds exhibiting gradual potentiometric changes near the end point, i. e., those having titration curves similar to those in Fig. 1. A colorimetric check of the normality of the perchloric acid reagent with potassium acid phthalate as a standard revealed no change; however, a potentiometric titration with the same standard revealed the source of the problem (Fig. 4). The electrolyte containing chloride ion apparently was diffusing into the titration medium at such a rate that there was a lessening of the changes in potential after the potassium acid phthalate end point. These changes had little effect on the values obtained from colorimetric or potentiometric standardization with potassium acid phthalate or the titration of other compounds with very sharp end

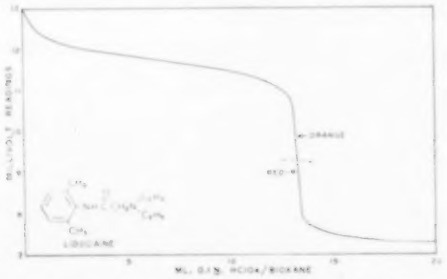

Fig. 3.—Potentiometric titration of lidocaine base in chloroform-acetonitrile (4:1) with perchloric acid in dioxane and with methyl red added as an indicator.

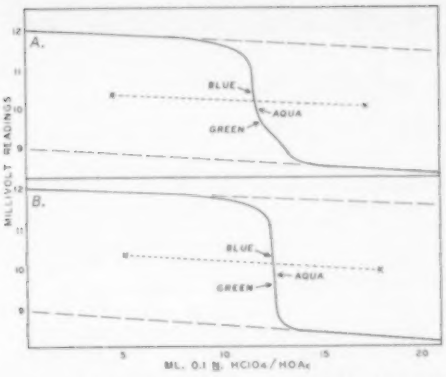

Fig. 4.—Potentiometric titration of potassium acid phthalate in glacial acetic acid with perchloric acid in glacial acetic acid, using combination electrode and with crystal violet added as an indicator. Titration *A*–curve obtained with excessive electrolyte flow. Titration *B*–normal curve. Calculated normalities of the perchloric acid titrant for curves *A* and *B* based on potassium acid phthalate titrated are 0.1001 and 0.1002, respectively.

points. In titrations where the change in potential at the end point was not sharp, such as in Fig. 1, this effect would blend into the end point of the compound causing high results not readily detectable in the usual potentiometric titration.

The substitution of another electrolyte such as potassium or ammonium nitrate which has been equilibrated with silver chloride has worked very well. Such an electrolyte which contains a much smaller amount of chloride should not cause difficulties, even if there is a fairly rapid diffusion of electrolyte into the titration mixture due to faulty electrode operation (Fig. 5). It is apparent that titrations in water-miscible, nonaqueous solvents such as glacial acetic acid are much less affected by water than by chloride ion. When mercuric acetate has been added to the glacial acetic acid titration medium, this chloride effect will not be present since the mercuric acetate effectively ties up the ionizable chloride.

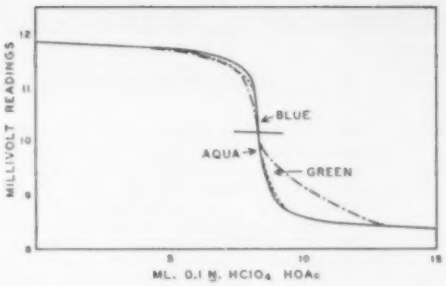

Fig. 5.—Potentiometric titration of potassium acid phthalate in glacial acetic acid with perchloric acid in glacial acetic acid with crystal violet added as an indicator. ——Nothing added, ----0.05 ml. of 10% potassium nitrate electrolyte added to the titration mixture, — - —0.05 ml. of 30%, 4 molar, potassium chloride added.

It is necessary, however, that the electrolyte be equilibrated with silver chloride or contain sufficient chloride ion, so that the silver chloride coating on the electrode wire is not removed by the electrolyte.

This difficulty of rapid diffusion of electrolyte has been experienced only once and was probably due to the excessive swelling of the silicone rubber stopper. Stoppers which are more resistant to nonaqueous solvents are currently being evaluated by the manufacturer and it is possible that they may be available soon. If prolonged use of the available combination electrode is being considered for glacial acetic acid or other water-miscible nonaqueous titration solvents, this excessive diffusion factor should be recognized and the possibility of selecting an alternative electrolyte should be considered. In normal intermittent use with three different combination electrodes, no electrode difficulty has been experienced.

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Examination of the Seeds of *Polanisia trachysperma*

By MAN MOHAN KOCHHAR and HENRY M. BURLAGE

The oil (16.31 per cent) extracted with petroleum ether from *Polanisia trachysperma* seeds has a yellowish color, musk-like odor, bland taste, and possesses drying properties. Saponification, acid, and iodine values, and other constants are reported. The study indicates that the oil consists of 85.8 per cent of unsaturated fatty acids, including 81.2 per cent of oleic acid and 1.9 per cent of linoleic acid. The saturated fraction (8.6 per cent) contained palmitic, lignoceric, and stearic acids. The infrared spectrum of the oil, the saturated, and unsaturated fractions indicated the usual characteristics of the saturated and unsaturated fatty acid fractions. The oil showed no significant antibiotic or growth-promoting substances.

POLANISIA TRACHYSPERMA T. and G. belongs to the family *Capparidaceae*. It is a small shrub and generally found in dry soils in summer and fall. It occurs from Iowa to British Columbia and also in Texas, Arizona, and Nevada.

The stem is simple; leaf-blades 3-foliolate; petioles longer than the leaflets; stoutish leaflet 1–4 cm. long; the blades elliptic, oval, obtuse, entire, sessile; racemes 3–10 mm. long; bracts 1-foliolate, ovate, 0.5–1 cm. long; sepals lanceolate, 5 mm. long, ciliate accuminate; petals 4, 8–10 mm. long; claws as long as notches blades, stamens long-exserted; capsules erect, linear, 4–6 cm. long, about twice as long as the pedicels; seed 2 mm. broad, dark brown, pitted, and wrinkled all over (1).

EXPERIMENTAL

Isolation of the Oil.—The quantity of seed was limited. The powdered seeds (455.5 Gm.) were macerated for seven hours with petroleum ether, packed into a glass percolator, and then percolated continuously until all of the soluble ingredients were extracted (2).

The petroleum ether was removed by distillation, and the oil was dried in a desiccator until the constant weight was obtained. The yield was 16.31%.

Physicochemical Properties of the Oil.—(*a*) The oil was immiscible with water, insoluble in alcohol, but was soluble in petroleum ether, absolute ether, and carbon tetrachloride. The yellowish oil had a bland taste similar to linseed oil.

(*b*) To determine if the oil was saponifiable, a sample was placed on the microscopic slide and a drop of alcoholic potassium hydroxide was added and the mixture protected with a cover glass and observed under a microscope. Soap crystals began to form within a few minutes (3).

(*c*) After fusion with metallic sodium tests showed the absence of sulfur, nitrogen, halogens, and phosphorus (4).

Specific Gravity.—The specific gravity (5) of the oil was determined by pycnometer method and by the Fisher-Davidson gravitometer, and it was found to be 0.9108 at 24°.

Drying Properties of the Oil.—To determine the drying properties of the oil the following procedure was adopted (6): a known weight of the oil was placed on a tared glass plate and was kept in a dust-free atmosphere for twenty-eight days. Linseed and sesame oils were used under similar conditions for purpose of control. Weights were recorded every third day. All the samples showed increase in weight and it was concluded that the oil showed drying properties.

Other properties of the Oil—The acid value (7) of the oil was found to be 3.69; saponificatioa value (8) was found to be 150.5; ester value (9) was found to be 146.6; iodine value (10) was ascertained to be 73.9; unsaponifiable matter (11) amounted to 2.9%; and thiocyanogen number (12) was 72.03.

Separation of Saturated and Unsaturated Fatty Acids of the Oil.—The lead ether salt method (13) was followed to effect this separation. The sample used was 20.0 Gm. of the oil. The saturated fatty acid fraction was 8.6% of the oil, with a melting point range of 48 to 50°. The unsaturated fraction was found to be 85.8%. This was confirmed by the thiocyanogen number method.

Determination of the Unsaturated Fatty Acids.—From thiocyanogen number and iodine values, the hypothetically pure glycerides in the *Polanisia trachysperma* seed oil may be calculated. This is based upon the fact that thiocyanogen $(SCN)_2$ reacts as a typical halogen and is selective towards compounds with isolated double bonds. It is believed that thiocyanogen radical first united with the double bond in oleic acid, then only one of the two double bonds of linoleic acid, and two out of three double bonds of linolenic acid, whereas iodine reacts with all double bonds in the unsaturated fatty acids. Since no linoleic acid was present in *Polanisia trachysperma* oil as established by paper chromatography, the following formula (14) was used to calculate the percentage of glycerides in the fatty acids.

$$S = 100 - (Z + Y)$$
$$Y = 1.246\ \text{I. V.} - 1.253\ \text{T. V.}$$
$$Z = 2.525\ \text{T. B.} - 1.348\ \text{I. V.}$$

where S = per cent saturated acid glycerides and unsaponifiable matter, Y = per cent linoleic acid glycerides, Z = per cent oleic acid glycerides, I. V. = iodine value, and T. V. = thiocyanogen value.

By substitution in the above, it was found that *Polanisia trachysperma* seed oil contained linoleic acid, 1.9%; oleic acid, 81.2 %; total, 83.1 %.

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Therefore, the saturated fraction along with an unsaponifiable matter was found to be

$$100-(1.9+81.2)=16.9\%$$

To ascertain the various acids in the saturated and unsaturated fractions of the fatty acids, paper chromatography was used.

Partition Chromatography of Saturated and Unsaturated Fatty Acids of the Oil.—The saturated and unsaturated fatty acids separated by lead-ether method were subjected to paper chromatography to distinguish between different acids present in those fractions.

A known weight of the fatty acid mixture was dissolved in petroleum ether (100 mg. was dissolved in 100 ml. of petroleum ether). This concentration was selected so that 5 λ represents 5 mcg. of the mixed saturated acids (15). Ascending and descending methods were tried, and it was found that the descending method gave better results. Special filter paper, grade 3054B, Schleicher and Schuell Co., was used. The filter paper was dipped into a solution of $KAl(SO_4)_2$, drained and then dried in an oven at 70°. The dried filter papers were pressed to eliminate any wrinkles and then cut into strips.

The solvent phase (petroleum ether, the stationary phase) was poured directly on the bottom of the jar and the jar was covered for about twelve hours in order to saturate the enclosed atmosphere. The mobile phase, a mixture of freshly distilled carbon tetrachloride: methanol: ammonia (81:18:1) was placed in a 9-in. Pyrex plate supported on the support rack. Sixty λ of the solution was applied on the paper strips. Control samples of known fatty acids under similar conditions were also applied on different strips and descending paper chromatography performed.

After fourteen hours the strips were removed, dried, and sprayed with 0.1% rhodamin B solution in 0.05*N* hydrochloric acid. The papers were then dried and examined under the ultraviolet light. The separated fatty acids showed up as definite spots which were marked with a pencil so that the R_f value could be determined.

The samples of saturated fatty acids from the oil of *Polanisia trachysperma* developed spots at R_f 0.54, R_f 0.67, and R_f 0.95. These results indicated that palmitic, lignoceric, and stearic acids were present.

With the unsaturated fraction spots at R_f 0.50, R_f 0.56, R_f 0.83, and R_f 0.95 were obtained indicating that there were at least four different acids present. Two of them were oleic and linoleic acids, as indicated by their R_f values (0.95 and 0.83, respectively). Two unidentified unsaturated fatty acids observed by paper chromatography procedure merit further investigation.

Infrared Spectrum of the Oil.—The oil of the seeds of *Polanisia trachysperma* was subjected to infrared spectroscopy to determine the various functional groups in the oil. It is the rotational and vibrational energy of a molecule that accounts for the complexity of its spectrum in the region of 2 to 15 μ (16).

The spectrum of the oil was compared with the known samples of linseed oil, and by this the characteristics of the compound were deduced. The infrared spectrum of the oil of *Polanisia trachysperma* showed a strong band at 5.8 μ and a second band at 6.9 μ. Five to seven microns represent a double bond region and this indicated that the oil has considerable acids containing a double bond. From 3.33 to 3.57 μ is the normal aliphatic or —OH group area, and the band at 3.5 μ indicated that the acids contained normal aliphatic chain and hydroxyl group attached to them. Another band appears at 7.3 μ indicating that the oil contained an isopropyl group. Another band was observed at 8.19 μ and this indicated an ester group (17).

A band at 8.7 μ was observed and this was indicative of the presence of C—O—C ether group, of the type R—CH_2—O—CH_2—R, and another band at 11 μ indicated the presence of RCH=CH_2 group (17).

Infrared spectrum of linseed oil showed that the bands at 3.5, 5.8, and 6.9 μ were very close to the oil of *Polanisia trachysperma*. This indicated that the oil had some structural characteristics similar to those of linseed oil.

The unsaturated fraction of the oil of *Polanisia trachysperma* showed two sharp bands at 5.9 and 6.85 μ (double bond region). These bands were very similar to those of oleic acid. It has been confirmed by paper chromatography that the oil contained oleic acid.

Absorption produced from carbonyl group is found from 4.5 to 6.5 μ. They are usually the strongest bands in this region (5.5 to 7 μ region is often called the double bond region) and are often the strongest in the spectrum. Any strong band between 5.5 and 5.9 μ is probably due to an acid-carbonyl bond (18).

Vibrations characteristics of the carbon-carbon double bonds are similar to that of carbon-nitrogen type, with one exception. The carbon-carbon double band vibrations usually result in such a small change in dipole moment that the intensity of infrared absorption is very low. A sharp band at 3.45 μ is due to the normal aliphatic chain. The band at 7.1 μ might be due to —CH=CH_2 group (19).

In the case of the saturated fraction of the oil of *Polanisia trachysperma* a sharp band at 3.5 μ was observed which was indicative of —CH normal aliphatic chain. Another sharp band at 5.9 μ was indicative of carboxylic acid. A band at 6.9 μ might be due to the CH_2CH_2— saturated group. These bands were very similar to the bands of palmitic and lignoceric acid which were confirmed by paper chromatography.

Other Studies on the Oil.[1]—The oil was diluted in 95% ethanol so that no more than 2% ethanol would be present in the final assay medium, and added aseptically to sterile assay medium in tubes. Control tubes not inoculated served to determine the extent of turbidity due to oil and not to bacterial growth. At a concentration of 600 γ per ml., the oil itself contributed to the turbidity of the culture medium. Over a range of 20 to 600 γ per ml., no inhibitory effect was obtained on the growth of *Escherichia coli* 9723 in a salts-glucose medium (20), or on the growth of *Lactobacillus arabinosus* 17 - 5, *Lactobacillus casei* 7469, *Streptococcus faecalis* 8043, and *Streptococcus lactis* 8039 in an amino acid, vitamin, purine, pyramidine, salts-glucose medium (21) or on the growth of *Leuconostoc mesenteroides* P-60

[1] These studies were conducted by the Clayton Foundation Biochemical Institute of the University of Texas

in the latter medium, as previously modified (22). Under the same testing conditions as the P-60 strain, *Leuconostoc mesenteroides* 8086 and *Leuconostoc citrovorum* 8081 were slightly but variably inhibited in their growth at concentrations of 600 μ per ml. of the oil. Under the same testing conditions as the P-60 strain, *Leuconostoc mesenteroides* 8086 and *Leuconostoc citrovorum* 8081 were slightly but variably inhibited in their growth at concentrations of 600 μ per ml. of the oil.

In the absence of biotin, oleic acid stimulates the growth of *L. arabinosus* 17 - 5 as a replacement for biotin. The oil at a concentration 10 times that of oleic acid showed activity in promoting the growth of *L. arabinosus* in the absence of biotin.

DISCUSSION

A comprehensive search of the literature indicated that no studies had been reported on the oil. In order to obtain the oil, the seeds were macerated and percolated in a glass percolator with petroleum ether.

The lead-ether salt method used to separate saturated and unsaturated fatty acids gave good results as indicated by duplicate procedures. The fractions separated by this method were analyzed by paper chromatography to find different fatty acids, and the result was compared with the known fatty acids.

Paper chromatography was used for the verification of the fatty acids. The method was very satisfactory, although the developing solution was very sensitive to light, temperature, and should be freshly prepared for every test. It should be discarded if liquid separates or becomes cloudy.

Infrared spectra were also run to ascertain if the oil contained certain functional groups which were also found in fatty acids such as oleic, linoleic, palmitic, lignoceric, and stearic acid.

Fractional crystallization method from solvent at low temperature was tried but did not prove to be satisfactory.

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Permeability of Excised Human Keratin to Lipid-Soluble Substances

By DALE E. WURSTER and ROBERT E. DEMPSKI

A method has been developed to follow the rate of penetration of lipid-soluble substances in excised, human keratin tissue. These substances include a long chain alcohol, unsaturated fatty acids, and cholesterol esters. There appears to be present in this keratin tissue a permeability gradient to these substances. The center layers of tissue have a slower rate of penetration to these substances than either the interior or exterior layers. Ether extraction does not destroy this effect. Using oleyl alcohol as a reference, data are presented which compare the rate of penetration of these substances. The effect of adsorption which greatly influences the penetration rate is also discussed.

THE PENETRATION of chemicals into and through the skin has been the object of a large number of investigations, many of which have been rather thoroughly covered in review articles (1–5). However, the elucidation of additional information in this important research field is of particular interest to both the pharmaceutical and cosmetic industries. More specifically, the penetration of the skin by such common constituents of dermatological preparations as fatty acids, alcohols, and esters requires further study. Several previous studies have been carried out on these type agents and also

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on ointment bases and oils (6–8); however, much of the work was qualitative in nature. The inherent disadvantages of some of the methods used in previous studies have been discussed by other authors (5, 9–11).

The purpose of the present investigation was to develop a method to follow the rate of penetration of lipid-soluble substances and to determine the effect of adsorption on this rate. In order to eliminate some of the variables present in past work, excised human callous tissue was used. This was employed to represent the outer layer of the epidermis which some workers believe is a barrier to skin penetration (12–15). Nevertheless, it is recognized that certain differences may exist between callous tissue and the normal keratin layer of the stratum corneum.

EXPERIMENTAL

Apparatus.—The apparatus employed to determine the permeability of the keratin tissue was a modification of a diffusion cell used in a previous study in this laboratory (16). The cell was modified as follows: Teflon gaskets were used in the joints to prevent leaking and two center aluminum washers were used rather than one. These were needed to form a rigid frame to hold the layers of keratin tissue; whereas, in the previous study, the barrier material was placed in the center opening of the single washer. The center opening of each washer which exposed the tissue to the solution on one side and solvent on the other had a diameter of 6 mm.

Preparation of Keratin.—In this study thin sections of keratin tissue were used. These were obtained from excised human callous tissue in the following manner. The intact callus was first completely hydrated in an atmosphere saturated with water vapor at room temperature for a period of three to four days. This hydrated tissue was then sectioned on a freezing stage microtome to a thickness of 100 μ. The water of hydration was then removed by storing over concentrated sulfuric acid for two days. Extraction with anhydrous ether was accomplished by a gentle shaking of each section of keratin with 25 ml. of ether for twenty-four hours.

Procedure.—Since it was found that the penetration rate varied both between individuals and skin areas, it was necessary to devise a method to control accurately the area of each section of tissue that was exposed to the solution containing the test substance. This was done in the following manner. A template was cut to the shape of each large piece of callus from which the individual layers were subsequently cut. This template was then placed on one washer and the outline of the tissue was carefully drawn on the washer. A section of tissue was placed within this outline and cemented into position. The second washer was then placed on top and the apparatus assembled. In this way it was possible to determine the permeability of each layer of a single piece of keratin. Thus, each layer represented a deeper section of a single area of a piece of intact callous tissue.

Exactly 50 or 60 ml. of a solution containing the lipid-soluble substance dissolved in purified *n*-heptane was introduced into one chamber of the diffusion cell with the simultaneous addition of an equal volume of solvent into the other chamber. The entire assembly was then placed in a constant temperature bath at 25° and subjected to mild continuous shaking. Periodically, samples were removed from each chamber and analyzed for the concentration of lipid-soluble material. The initial concentration of the solutions employed was 20×10^{-6} moles per ml. unless otherwise indicated.

The quantitative determination of oleyl alcohol, oleic acid, linoleic acid, linolenic acid, and cholesteryl oleate was accomplished by ultraviolet spectrophotometry.

The cholesteryl acetate was assayed by means of a modification of a previously described colorimetric method (17). In this assay 1 ml. of the heptane solution was mixed with 2 ml. of anhydrous ether and 1 ml. of hydroxylamine reagent. This was allowed to stand for thirty minutes. Then 5 ml. of ferric perchlorate reagent was added and the absorbance read immediately against a reagent blank with a colorimeter at a wavelength of 520 mμ.

RESULTS AND DISCUSSION

The results indicate the permeability of keratin tissue varies among individuals, among skin areas, and also varies among the individual layers from the same piece of callous tissue. In order to determine the rate of penetration, permeability constants, K, were calculated from the following equation. The derivation of this equation is given in a previous communication from this laboratory (16).

$$\log (C_0 - 2C_b) = (-2K/2.303)t + \log C_0$$

By determining C_0, C_b, and t experimentally and plotting log $(C_0 - 2C_b)$ as a function of time in days, a straight line is obtained, the slope of which is equal to $-2K/2.303$. A sample plot is shown in Fig. 1. The numbers in parentheses represent the layer of tissue. Here the difference in rate between the layers of tissue is obvious. In some of the following graphs and tables it can be observed that some tissue layers have been omitted. These were layers where keratin sections were not obtained in the intact form or where the cells were obviously leaking and the results were invalid.

The rate of penetration varies greatly from layer to layer of tissue; however, when the permeability constant is plotted as a function of the layer of tissue, there appears to be present a permeability gradient, as illustrated in Fig. 2. The lowest number represents the outermost layer of tissue that could be used.

The observation that the various layers of a single area of callous tissue show different rates of penetration to a specific penetrant was first made on tissue sections which had not been extracted with ether. When the keratin sections were extracted with ether prior to exposure to the penetrant the effect was not destroyed. All of the comparative data presented in this paper deals with tissue sections that were extracted with ether.

In Table I the data obtained for a single piece of callous tissue which was sectioned into layers and the permeability rates of oleyl alcohol and cholesteryl

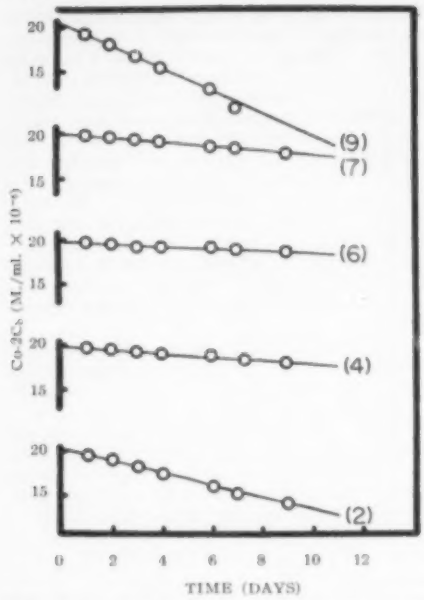

Fig. 1.—Sample plots for the penetration of oleyl alcohol through various layers of ether-extracted tissue.

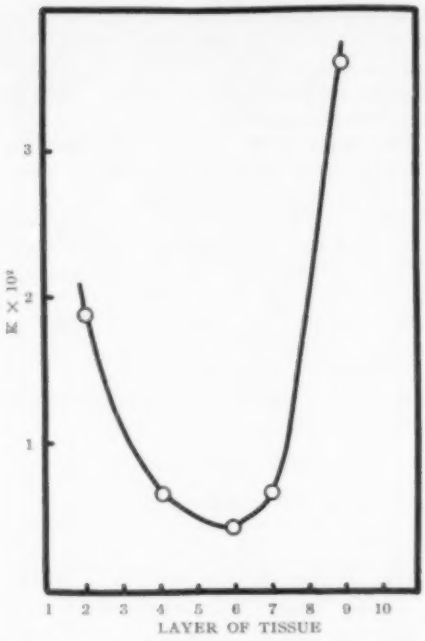

Fig. 2.—A plot showing the permeability constant *versus* layer of ether-extracted tissue.

TABLE I.—DATA FOR THE PENETRATION OF CHOLESTERYL ACETATE USING OLEYL ALCOHOL AS A REFERENCE

Layer of Tissue	Oleyl Alcohol (M/C_0 ml. × 10^{-6})	Oleyl Alcohol $K \times 10^2$	Cholesteryl Acetate (M/C_0 ml. × 10^{-6})	Cholesteryl Acetate $K \times 10^2$
1	20.25	0.550	...	...
2	...	...	20.00	0.829
3	20.25	0.438	...	...
4	...	...	20.00	0.206
5	20.25	0.299	...	...
6	...	...	20.00	0.153
8	...	...	20.00	0.102
9	20.25	0.117	...	...
10	...	...	20.00	0.286
11	20.25	0.256	...	...
12	...	...	20.00	0.542

TABLE II.—COMPARISON OF AVERAGE PERMEABILITY CONSTANTS OF VARIOUS PENETRANTS WITH OLEYL ALCOHOL USING ETHER-EXTRACTED CALLOUS TISSUE

Test Penetrant	Average Permeability Constant, $K \times 10^2$	Average Permeability Constant for Oleyl Alcohol on Same Piece of Ether-Extracted Tissue, $K \times 10^2$
Oleyl alcohol (non-extracted tissue)	0.340	...
Cholesteryl acetate	0.313	0.332
Cholesteryl oleate	0.062	0.208
Oleic acid	0.310	0.650
Linolenic acid	0.021	0.133
Linoleic acid	0.009	0.229

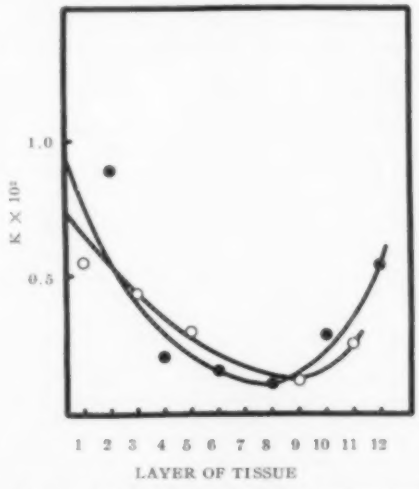

Fig. 3.—A plot showing the rate of penetration of oleyl alcohol and cholesteryl acetate through alternate layers of tissue. ○, Oleyl alcohol; ●, cholesteryl acetate.

acetate determined on the individual layers are shown. All callous tissue investigated with the various penetrants was handled in this manner. As previously stated, in each case oleyl alcohol served as the reference substance. When the experimental data were plotted (permeability constant *versus* tissue layer), as shown in the example given in

Fig. 3, the lower penetration rates of the center layers were readily apparent. From this figure it appears that the permeability rates of these two substances are quite similar.

In order to compare the penetration rates of the various penetrants the permeability constants of the individual layers were averaged. Table II contains the average permeability constants for the various penetrants tested. Column 2 contains the average permeability constants obtained for the test penetrants and column 3 contains the average values obtained for oleyl alcohol on the alternate layers of the same piece of keratin tissue. Although the penetration rate of cholesteryl acetate was about the same as oleyl alcohol, the rate for the larger cholesteryl oleate molecule was much slower (approximately one-third).

Previously in this study (18) it was shown that certain fatty acids including linoleic, linolenic, and 2-hydroxystearic were adsorbed by powdered, excised, human keratin tissue, whereas oleic acid was not adsorbed. In an attempt to determine the effect of adsorption on the rate of penetration, oleic, linoleic, and linolenic acids were tested according to the method described above. Again in Table II it can be observed that both linolenic and linoleic acids have very low rates of penetration. Subsequent adsorption experiments have shown that neither oleyl alcohol, cholesteryl acetate, or cholesteryl oleate are adsorbed by keratin. Thus, it appears that those substances which interact strongly enough with keratin to be adsorbed have low penetration rates.

Various skin structures including the transition layer have been mentioned as barriers to the penetration of chemical agents through the intact skin (19, 20). It appears to be particularly significant that this study has indicated the presence of a barrier in excised human callous tissue which may possibly also exist in the normal keratin layer.

A subsequent paper will include penetration data on additional lipid-soluble substances and mixtures.

SUMMARY

A quantitative method for determining the rate of penetration of various lipid-soluble substances in excised human callous tissue has been developed.

On the basis of the permeability constants obtained in this study all test penetrants showed lower penetration rates in the center layers of excised human keratin tissue than in either the exterior or interior layers.

Experimental evidence indicates that the relative penetration rates for the test penetrants in keratin tissue are in the following order: oleyl alcohol ~ cholesteryl acetate > oleic acid > cholesteryl oleate > linolenic acid > linoleic acid.

Substances such as linolenic and linoleic acids which are adsorbed by keratin appear to have slower penetration rates in keratin tissue than substances of comparable molecular size which are not adsorbed.

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Studies on Protective Coating IX

Polyvinylpyridine Derivatives

By ISAMU UTSUMI, TADAO IDA, SHOZI TAKAHASHI, and NORIO SUGIMOTO

Polymers of vinylpyridine and of its derivatives and copolymers of vinylpyridine with other series of vinyl compounds were experimentally synthesized. The purpose of the synthesis was to obtain such protective coating agents exhibiting protective activity on drugs and solubility in gastric juice. Solubility, water vapor permeability through the film, and viscoelasticity were tested for each compound. The practical coatings on tablets were also carried out and disintegration time of coated tablets and shelf life of active ingredients were subsequently investigated. All the compounds showed excellent properties as protective coating agents.

IN MANY CASES drugs will become moist, colored, and decompose with a loss of their efficacies if they are not protected from atmospheric effects such as moisture, oxygen, carbon dioxide, light, etc. If the sugar coating or gelatin coating procedure is carried out in the usual manner, the chances are that such drugs having a great affinity for moisture will absorb some of the water from the syrup or gelatin film. This will cause sugar coated tablets to develop cracks which continue to grow, thus making the tablets fissured or mottled and spotted in appearance.

With such drugs, it is very important to take special measures to eliminate or curtail these influences as much as possible. For this reason, a moisture or oxygen-resistant protective coating appears valuable to produce a stable product.

Shellac, nitrocellulose, acetylcellulose, and waxes have been widely used as coating agents because of their water-resistant properties. Although protection of tablets from moisture can be completely obtained by these coating agents, the active ingredients are not absorbed from the gastrointestinal tract satisfactorily because of the incomplete dissolution of the coating agents in the body.

Another disadvantage of these coating agents is that a thin coat can not give sufficient protection against moisture.

Considering these limitations, the authors have synthesized some organic compounds such as aminoderivatives (1, 2), or amino acid esters (3, 4) of cellulose derivatives, saccharides, and polyhydric alcohols; polyvinylamine derivatives (5) and polyvinylamino acetal derivatives (6) in the hope of obtaining the properties of strong water-resisting power and enough solubility in the gastric juice. The latter property is essential to expedite effective absorption of a drug from the digestive tract. The applicability of these compounds has already been discussed in the former reports (7, 8) in which 21 kinds of useful polymers were found to be practically employable as coating agents on a manufacturing scale.

In this paper, experimental work concerning polyvinylpyridine and the polymers of its alkyl derivatives and its copolymers with other vinyl compounds are presented.

Polymerization of vinylpyridine derivatives has been investigated by many researchers (9–12) and copolymerization of vinylpyridine derivatives with other vinyl compounds or butadiene derivatives has also been studied (13–15).

However, no report has ever been presented concerning application of these compounds as coating agents for medical use.

Homopolymers of four vinylpyridines: 2-vinylpyridine (2VP), 4-vinylpyridine (4VP), 2-methyl-5-vinylpyridine (MVP), and 2-vinyl-5-ethylpyridine (VEP), were first synthesized using either catalyst of benzoylperoxide (BPO) or potassium persulfate ($K_2S_2O_8$), and copolymers of each compound with styrene (St), vinylacetate (VAc), methylacrylate (MA), and acrylonitrile (AN) were also synthesized by the same procedure.

The applicability of these compounds as protective coating agents was extensively investigated.

EXPERIMENTAL

Materials.—Each monomer was rectified before using. The boiling point of each monomer is shown as follows: 2-vinylpridine, 71° (30 mm. Hg); 4-vinylpyridine, 65° (15 mm. Hg); 2-methyl-5-vinylpyridine, 99–100° (50 mm. Hg); 2-vinyl-5-ethylpyridine, 95–97° (18 mm. Hg); styrene, 81–82° (100 mm. Hg); vinylacetate, 71–72°; methylacrylate, 80–81°; acrylonitrile, 76–77°.

Synthesis of Polymers.—*Homopolymers.*—Two methods were used for synthesizing homopolymers: (*a*) Each vinylpyridine monomer was dissolved in methanol and then BPO was added as a catalyst. The solution was heated to cause the polymerization.

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TABLE I.—POLYMERIZATION OF VINYLPYRIDINE DERIVATIVES

Monomer, Gm.		Catalyst, Gm.		Solvent, Gm.		Temperature, °C.	Time, hr.	Yield— Gm.	Yield— %	Intrinsic Viscosity
2VP	5.3	BPO	0.1	MeOH	1.6	50	3.7	2.5	47.2	0.50
	19.5	$K_2S_2O_8$	0.4	10% H_2SO_4	184.0	50	10.3	18.5	95.0	0.69
4VP	5.3	BPO	0.1	...		50	2.0	5.0	94.4	0.26
	5.3	$K_2S_2O_8$	0.1	10% H_2SO_4	50.0	50	53.5	4.4	83.1	0.26
MVP	11.9	BPO	0.2	MeOH	4.0	50	23.4	9.0	75.6	1.02
	23.8	$K_2S_2O_8$	0.4	10% H_2SO_4	200.0	50	49.0	22.6	95.0	1.04
VEP	6.4	BPO	0.1	EtOH	10.0	Reflux	7.0	3.8	59.3	0.14
	6.7	$K_2S_2O_8$	0.05	10% H_2SO_4	50.0	50	1.0	6.5	97.0	2.04

TABLE II.—COPOLYMERIZATION OF 2-VINYLPYRIDINE WITH OTHER VINYL COMPOUNDS

2VP, Gm.	Comonomer, Gm.		Catalyst, Gm.		Solvent, Gm.		Emulsifier, Gm.	Temperature, °C.	Time, hr.	Yield— Gm.	Yield— %	Intrinsic Viscosity	2VP Content, Mol. %
5.3	VAc	8.6	BPO	0.4	MeOH	4	...	55	23.5	3.7	26.6	0.11	98.3
4.3	AN	2.2	BPO	0.2	MeOH	4	...	55	4.8	5.0	77.0	0.60	83.3
5.3	MA	4.3	BPO	0.2	MeOH	4	...	55	11.5	7.3	76.0	0.54	81.9
5.3	St	5.2	BPO	0.2	MeOH	4	...	55	46.3	7.4	69.8	0.45	53.3
10.6	VAc	8.6	$K_2S_2O_8$	0.4	10% H_2SO_4	100	0.4	50	52.4	11.5	59.9	0.29	70.8
2.0	AN	1.1	$K_2S_2O_8$	0.08	10% H_2SO_4	20	0.04	60–70	3.0	1.7	55.0	0.45	91.8
2.0	MA	1.7	$K_2S_2O_8$	0.08	10% HCl	8	0.04	60–70	3.0	1.2	32.5	0.22	93.4
10.6	St	10.5	$K_2S_2O_8$	0.2	10% H_2SO_4	100	0.4	50	33.4	18.7	88.6	1.87	75.5

TABLE III.—COPOLYMERIZATION OF 4-VINYLPYRIDINE WITH OTHER VINYL COMPOUNDS

4VP, Gm.	Comonomer, Gm.		Catalyst, Gm.		Solvent, Gm.		Emulsifier, Gm.	Temperature, °C.	Time, hr.	Yield— Gm.	Yield— %	Intrinsic Viscosity	4VP Content, Mol. %
5.3	VAc	4.3	BPO	0.1	MeOH	4	...	50	45.8	5.3	55.2	0.11	89.8
5.3	AN	2.7	BPO	0.1	...		...	50	0.3	4.0	50.0	0.10	81.6
5.3	MA	4.3	BPO	0.1	...		...	50	6.0	2.0	20.8	0.29	74.7
5.3	St	5.2	BPO	0.2	MeOH	4	...	50	6.0	6.5	61.8	0.41	70.8
5.3	VAc	4.3	$K_2S_2O_8$	0.1	10% H_2SO_4	50	0.1	50	7.3	5.5	57.3	1.24	90.4
10.6	AN	5.3	$K_2S_2O_8$	0.2	10% H_2SO_4	100	0.2	50	6.0	12.5	78.6	0.45	80.8
5.3	MA	4.3	$K_2S_2O_8$	0.1	10% H_2SO_4	50	0.2	50	7.5	5.7	59.4	1.16	82.6
5.3	St	5.2	$K_2S_2O_8$	0.1	10% H_2SO_4	50	0.1	50	7.3	9.6	91.3	0.66	51.9

The polymer thus obtained was dissolved in diluted hydrochloric acid and subsequently the polymer was separated by the addition of caustic alkali. (*b*) Diluted hydrochloric acid or diluted sulfuric acid solution of each vinylpyridine monomer was polymerized by using potassium persulfate as a catalyst while heating. The solution was subsequently diluted with water and then the polymer was separated out by caustic alkali. Table I shows the condition of its reaction.

Copolymers.—Two procedures were used for making copolymers:

(*a*) A mixture of vinylpyridine monomer and each comonomer was dissolved in methanol and polymerized by heating in the presence of BPO as a catalyst. (*b*) Emulsion polymerization was successively carried out by using polyoxyethylene monostearate and potassium persulfate as an emulsifying agent and a catalyst, respectively, in a diluted sulfuric acid solution of the vinylpyridine monomer and comonomer. Tables II, III, IV, and V show the conditions of the reaction.

Composition of Copolymers.—The composition of each copolymer was determined in 1% hydrochloric acid solution by ultraviolet absorption (16, 17). The ultraviolet absorption maxima of poly-2VP (P2VP), poly-4VP (P4VP), and both poly-MVP (PMVP) and poly-VEP (PVEP) are 262.5, 254, and 269.5 mμ, respectively. The determined values are shown in Tables II, III, IV, and V.

Water Vapor Permeability of Films.—An increase in the amount of water permeated through the film per twenty-four hours in a room controlled at 25° and 92% R. H. was measured in accordance with the method specified in ASTM, E 96-53T. The amount of permitted water vapor was subsequently calculated using the following formula:

$$Q = \frac{W \cdot L}{S} \text{ (Gm. cm./cm.}^2 \text{ 24 hr.)}$$

where W = increased weight, Gm.; L = thickness of film, cm.; and S = area of film, cm.^{2}.

The polymer-films were prepared by the following procedure: a solution of protective coating agent dissolved in organic solvent was poured onto the surface of mercury, dried in air, and further dried thoroughly in an anhydrous phosphoric acid desiccator under reduced pressure.

Viscoelasticity of Films.—The bending method was applied for the determination of the viscoelas-

TABLE IV.—COPOLYMERIZATION OF 2-METHYL-5-VINYLPYRIDINE WITH OTHER VINYL COMPOUNDS

MVP, Gm.	Comonomer,	Gm.	Catalyst,	Gm.	Solvent,	Gm.	Emulsifier, Gm.	Temperature, °C.	Time, hr.	Yield— Gm.	Yield— %	Intrinsic Viscosity	MVP Content, Mol. %
23.8	VAc	34.4	BPO	0.9	MeOH	16	...	50	19.2	17.0	29.2	0.38	97.2
15.4	AN	11.0	BPO	0.2	MeOH	10	...	50	6.3	15.0	57.0	0.60	97.4
23.8	MA	17.2	BPO	0.4	MeOH	16	...	50	15.6	21.0	51.3	1.05	90.8
23.8	St	20.8	BPO	0.6	MeOH	16	...	50	64.0	24.0	53.8	0.66	59.8
9.6	VAc	6.8	$K_2S_2O_8$	0.3	10% H_2SO_4	80	0.16	25	20.0	8.2	50.0	0.59	84.1
9.6	AN	4.4	$K_2S_2O_8$	0.3	10% H_2SO_4	80	0.16	60	3.0	8.9	63.7	1.09	46.5
12.0	MA	8.5	$K_2S_2O_8$	0.4	10% H_2SO_4	10	0.2	60	3.0	8.2	13.6	1.12	52.3
25.8	St	20.8	$K_2S_2O_8$	0.4	10% H_2SO_4	200	0.8	50	7.6	27.2	61.0	1.08	92.5

TABLE V.—COPOLYMERIZATION OF 2-VINYL-5-ETHYLPYRIDINE WITH OTHER VINYL COMPOUNDS

VEP, Gm.	Comonomer,	Gm.	Catalyst,	Gm.	Solvent,	Gm.	Emulsifier, Gm.	Temperature, °C.	Time, hr.	Yield— Gm.	Yield— %	Intrinsic Viscosity	VEP Content, Mol. %
6.7	VAc	4.3	BPO	0.2	...		...	70	5	6.8	61.8	0.16	73.3
40.0	AN	15.9	BPO	0.7	MeOH	25	...	55	2.6	47.1	84.2	1.21	55.9
26.6	MA	17.2	BPO	0.5	MeOH	20	...	50	24	37.3	85.0	0.83	80.8
200.0	St	100.0	BPO	5.0	MeOH	80	...	55	40	28.5	95.0	0.80	57.2
6.7	VAc	4.3	$K_2S_2O_8$	0.1	10% H_2SO_4	25	0.1	Room temp.	9	5.2	47.3	0.72	81.2
6.7	AN	2.7	$K_2S_2O_8$	0.1	10% H_2SO_4	50	0.1	30–40	3	6.5	69.1	0.75	50.5
6.7	MA	4.3	$K_2S_2O_8$	0.1	10% H_2SO_4	50	0.2	30–40	3	5.4	49.2	0.31	77.0
6.7	St	5.2	$K_2S_2O_8$	0.2	10% H_2SO_4	50	0.1	40	2	8.0	63.2	1.05	55.6

TABLE VI.—DATA OF COATING FOR AGING TEST

Tablet	Coating Agent	Solvent	Concentration, w/v %	Thickness of Film, μ
Acetylsalicylic acid tablet	P-VEP	CCl_4	7	119.0
	VEP-AN	Benzene	10	127.5
	VEP-St	CCl_4	10	80.8
	MVP-St	Benzene	10	57.3
Acetylsalicylic acid-antipyrine-phenacetin tablet	VEP-AN	Benzene	10	84.3
	VEP-St	CCl_4	10	112.6
Vitamin B_1-C tablet	VEP-AN	Benzene	10	127.5
	VEP-St	CCl_4	10	80.8

ticity of films. This method consists of a film fixed in a clamp free state and loaded at a certain distance from the free end. Change in bending at the free end along with the progress of time was measured with the calibrating microscope. The creep was observed in such a manner and from these results the phenomenon was analyzed because it was supposed to correspond to 4-element model.

The modulus of elasticity, coefficient of viscosity, and retardation time in each copolymer were calculated from the analyzed results. All measurements were carried out in a room kept at a constant temperature of 25° and a humidity of 25% R. H.

The films used in this experiment were made by the same procedure used in the permeability test of films.

Disintegration Test.—The protective coating was applied on starch-lactose tablets by means of dipping method. These tablets were composed of starch 45%, lactose 43%, dextrin 7%, and talc 5%, and had a diameter of 8 mm., a radius of curvature of 7.5 mm., and a thickness of 4 mm. After having completed the coating, the disintegration time of the coated tablets was observed in distilled water and artificial gastric juice in accordance with the method specified in U. S. P. XV. The thickness of a film was calculated from the amount of each coating agent used.

Aging test.—Formulas of tablets used in the experiment for aging test are shown below:

(*a*) *Acetylsalicylic Acid Tablet.*—Acetylsalicylic acid, 115 mg.; starch, 15 mg.; total, 130 mg.

(*b*) *Acetylsalicylic Acid-Antipyrine-Phenacetin Tablet.*—Acetylsalicylic acid, 50 mg.; antipyrine, 30 mg.; phenacetin, 30 mg.; starch, 38 mg.; magnesium stearate, 2 mg.; total, 150 mg.

(*c*) *Vitamin B_1-C Tablet.*—Vitamin B_1 mononitrate, 5 mg.; vitamin C, 30 mg.; starch, 20 mg.; lactose, 55 mg.; polyvinylpyrrolidone, 5 mg.; talc, 4 mg.; magnesium stearate, 1 mg.; total, 120 mg.

The tablets formulated by (*a*) and (*b*) were compressed by a slugging method, but only the tablet (*c*) was carried out by the wet granulation method in which methanol was used as binding solution.

Dipping method was applied on all tablets for application of the protective coating, but dusting powder was not used at all. Table VI shows the data concerning protective coating.

Tablets coated were stored in a room kept at constant temperature and humidity at 37° and 85% R. H., respectively, and the change in the deterioration of active ingredients was observed as follows; in tablets (*a*) and (*b*) the content of salicylic acid formed by the decomposition of acetylsalicylic acid was measured quantitatively by means of its ultraviolet absorption (18), and in the tablets (*c*) the assay was performed by measuring

TABLE VII.—SOLUBILITY OF POLYMERS AND COPOLYMERS

Coating Agent	Solvent[a] MeOH	EtOH	Acetone	$CHCl_3$	EtOAc	Benzene	CCl_4	Dioxane	Ether	Petroleum Ether	Distilled Water	Artificial Gastric Juice
P·2VP	S	S	S	S	S	S	I	S	I	I	I	S
2VP-VAc	S	S	S	S	SS	SS	I	S	I	I	I	S
2VP-AN	S	S	S	S	S	S	I	I	I	I	I	S
2VP-MA	S	S	SS	S	SS	SS	I	I	I	I	I	S
2VP-St	S	S	S	S	S	S	I	S	I	I	I	S
P·4VP	S	S	SS	S	I	G	G	G	I	I	I	S
4VP-VAc	S	S	SS	S	I	G	G	G	I	I	I	S
4VP-AN	S	S	SS	S	I	I	I	G	I	I	I	S
4VP-MA	S	S	I	G	I	I	I	I	I	I	I	S
4VP-St	S	S	SS	S	I	SS	G	G	I	I	I	S
P·MVP	S	S	S	S	S	SS	I	S	I	I	I	S
MVP-VAc	S	S	S	S	S	SS	I	S	I	I	I	S
MVP-AN	S	S	S	S	SS	I	I	S	I	I	I	S
MVP-MA	S	S	G	S	G	SS	I	S	I	I	I	S
MVP-St	S	S	S	S	S	S	S	S	I	I	I	S
P·VEP	S	S	S	S	S	S	S	S	I	I	I	S
VEP-VAc	S	S	S	S	S	S	S	S	I	I	I	S
VEP-AN	S	S	S	S	S	S	SS	S	I	I	I	S
VEP-MA	S	S	S	S	S	S	S	S	I	I	I	S
VEP-St	S	S	S	S	S	S	S	S	I	I	I	S

[a] S, Soluble; SS, slightly soluble; G, gelatinized or swollen; I, insoluble.

the contents of vitamin C and B_1 by using indophenol titration method and thiochrome fluorimetry.

Toxicity Test.—Each coating agent, 10–20% solution, in artificial gastric juice or suspension of each coating agent added with a small amount of CMC was given orally to dd-mice, weighing 20 ± 1 Gm. by means of stomach tube. The lethal number of mice was observed seventy-two hours after the administration. The dose administered was the maximum dose which could be given.

RESULTS AND DISCUSSION

Solubility.—As shown in Table VII all vinylpyridine homopolymers and copolymers were insoluble in water but very soluble in acid and in artificial gastric juice. Besides, all compounds produce transparent films which are soluble in almost all kinds of organic solvents.

It appears that all of the polymers possess the essential properties necessary for use as protective coating agents.

Since each polymer of VEP is soluble in all organic solvents except in ether and petroleum ether series, the hoped for advantage is that the choice of solvent should be possible according to the drugs to be coated. This superiority has never been recognized in any kind of coating agents synthesized before by us, and also in enteric coating agents such as cellulose acetate phthalate.

Water Vapor Permeability and Viscoelasticity of Films.—The water vapor permeability of films of polymers, as shown in Table VIII, was in the order ranging from 10^{-4} to 10^{-5} units. Although only the polymers of 4VP exhibited a slightly higher value, all of the others showed much lower values compared with that of shellac which was used as a control. Particularly in the polymers of VEP and the copolymers of styrene with each VP, the water vapor permeability was so low that these compounds are obviously excellent protective coating agents.

TABLE VIII.—WATER VAPOR PERMEABILITY AND VISCOELASTICITY OF FILMS

Coating Agent	Permeability, $\times 10^{-4}$ Gm. cm./cm.^{2} 24 hr.	Elasticity, $\times 10^{10}$ dyne/cm.^{2}	Retardation Time, $\times 10^3$ sec.	Viscosity, $\times 10^{14}$ Poise
P·2VP	0.769	34.40	6.22	∞
2VP-VAc	0.965	33.95	1.99	60.17
2VP-AN	0.489	36.59	7.23	54.88
2VP-MA	1.042	0.22	2.32	0.12
2VP-St	0.207	33.63	6.38	68.28
P·4VP	5.972	116.57	3.42	87.93
4VP-VAc	5.265	46.72	10.71	103.58
4VP-AN	3.728	49.96	4.58	77.04
4VP-MA	6.766	89.47	7.41	142.07
4VP-St	0.530	232.58	4.90	588.16
P·MVP	2.314	26.76	10.53	68.80
MVP-VAc	2.064	11.81	15.79	5.86
MVP-AN	1.199	50.57	16.22	32.18
MVP-MA	1.538	28.91	14.29	29.23
MVP-St	0.646	3.08	9.09	2.84
P·VEP	0.551	16.07	16.55	0.12
VEP-VAc	0.620	0.81	24.74	0.08
VEP-AN	0.519	31.94	13.04	2.24
VEP-MA	0.472	21.03	8.70	11.69
VEP-St	0.320	16.12	15.58	4.74
Shellac	8.813			

As shown in Table VIII most of elasticity and viscosity of films were 10^{10}–10^{12} dyne/cm.^{2} and 10^{13}–10^{14} poises, respectively, although there were only two or three exceptions. Therefore, nearly all of the polymers seemed to be suitable as coating agents.

Disintegration of Polymer Coated Tablets.—The results of disintegration test of starch-lactose tablets are shown in Table IX. All coated tablets were stable in the distilled water and easily disintegrated in artificial gastric juice.

TABLE IX.—DISINTEGRATION TEST

Coating Agent	Coating Solution—Solvent	Concentration, %	Thickness of Film, μ	Disintegration Time—Distilled Water, Hr.	Disintegration Time—Artificial Gastric Juice, min.
P-2VP	MeOH	20	202	4	5–10
2VP-VAc	MeOH	20	205	2–3	5–10
2VP-AN	MeOH	20	201	4	5–15
2VP-MA	MeOH	20	209	2–3	5–10
2VP-St	MeOH	20	180	4	5–15
P-4VP	MeOH	20	222	4	7–10
4VP-VAc	MeOH	20	235	4	7–10
4VP-AN	MeOH	20	220	4	7–10
4VP-MA	MeOH	20	240	4	7–10
4VP-St	MeOH	20	201	4	7–10
P-MVP	MeOH	10	151	4	8–10
MVP-VAc	MeOH	10	154	2–3	9–10
MVP-AN	MeOH	10	156	4	15–16
MVP-MA	MeOH	10	112	4	15–16
MVP-St	MeOH	10	148	4	8–10
P-VEP	CCl_4	10	159	4	5–10
VEP-VAc	CCl_4	10	220	4	5–10
VEP-AN	CCl_4	10	212	4	5–10
VEP-MA	CCl_4	10	207	4	5–10
VEP-St	CCl_4	10	150	4	5–10

All polymers and copolymers, therefore, perfectly protect labile ingredients from air, light, and moisture while displaying desirable effects by disintegrating rapidly in the stomach.

Protective Effect on Active Ingredients.—Protective coatings with the polymers were carried out on acetylsalicylic acid tablets, acetylsalicylic acid-antipyrine-phenacetin tablets, and vitamin B_1-vitamin C tablets. On these tablets, the aging tests were attempted. Figures 1–4 show the results.

Polymer coated tablets were apparently stabilized when compared with the decomposition rate of uncoated tablets used as a control.

Toxicity.—Table X shows the result of the oral acute toxicity test of the protective coating agents. In all the mice, no deaths were caused by the coating agents seventy-two hours after the administration.

It is, therefore, concluded that all the polymers and copolymers for protective coating agents do not possess any toxicity.

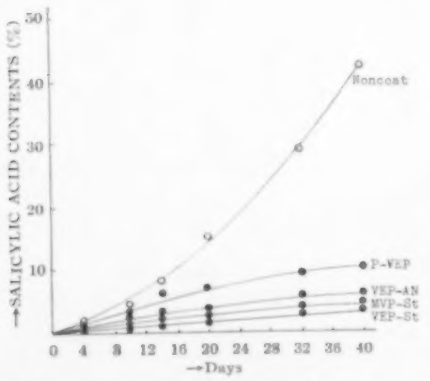

Fig. 1.—Aging of acetylsalicylic acid in acetylsalicylic acid tablets at 37°, 85% R. H.

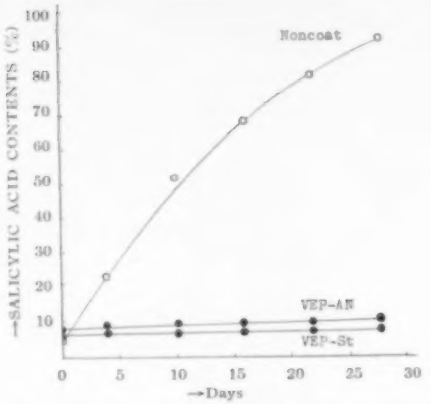

Fig. 2.—Aging of acetylsalicylic acid in acetylsalicylic acid-antipyrine-phenacetin tablets at 37°, 85% R. H.

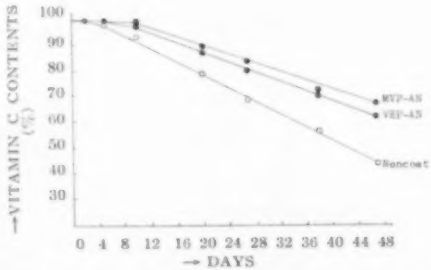

Fig. 3.—Aging of vitamin C in vitamin B_1-C tablets at 37°, 85% R. H.

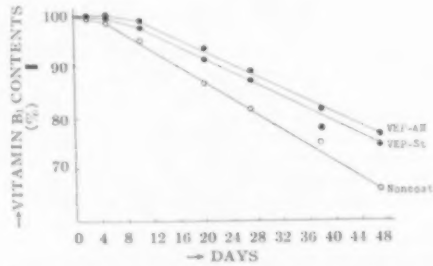

Fig. 4.—Aging of vitamin B_1 in vitamin B_1-C tablets at 37°, 85% R. H.

TABLE X.—ACUTE TOXICITY TEST IN MICE[a]

Dosed Animal No.	Protective Coating Agent	Dose, mg./Kg.
6	P-2VP	3,000
6	P-4VP	1,500
6	P-MVP	2,000
6	P-VEP	5,000
6	VEP-VAc	5,000
10	VEP-St	3,000
6	VEP-AN	2,000
6	MVP-MA	3,000

[a] No deaths seventy-two hours after administration.

SUMMARY

1. Polymers of 2-vinyl, 4-vinyl, 2-methyl-5-vinyl, and 2-vinyl-5-ethylpyridine, and copolymers of these vinyl compounds with other series of vinyl compounds were synthesized to obtain new protective coating agents.

2. These polymers and copolymers are insoluble in water. However, all are soluble in artificial gastric juice and in most of the organic solvents and produce transparent films. Water vapor permeability through the film is very low. Furthermore, it was found that these compounds possess an adequate viscoelasticity as protective coating agents.

3. By the coating of tablets with these polymers and copolymers, drugs are remarkably stabilized so that deterioration of active ingredients during storage is reduced. Moreover, because the films coated on tablets are easily soluble in gastric juice, the coated tablets are disintegrated freely followed by displaying definite effects of the drugs.

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Pharmacology of 3,5-Diphenyl-1,2,4-triazole

By S. M. RAINES, J. W. MARTIN, and D. B. MEYERS

Pharmacological studies showed 3,5-diphenyl-1,2,4-triazole to have both stimulating and depressing effects on the C. N. S. of unanesthetized rats and mice. In moderate doses it lowered blood pressure and stimulated intestinal muscle in anesthetized cats. Neural muscular transmission was blocked in rats and frogs. An inhibition of serum cholinesterase activity by the drug was demonstrated.

THE PURPOSE of this investigation was to study the pharmacological properties of 3,5-diphenyl-1,2,4-triazole (3,5-DPT). Although monophenyl derivatives of triazoles have been shown to be physiologically active, a review of the literature indicated a lack of pharmacological experimentation on simple diphenyl substituted derivatives. However, the syntheses of many such agents have been reported.

In the earliest reported studies of phenyl triazoles, Pellizzari (1) observed that 4-phenyl-1,2,4-triazole produced convulsions in frogs and dogs. Gibson, *et al.* (2), confirmed these studies and further showed that 1- and 4-phenyl-1,2,4-triazole had qualitatively opposing effects on the central nervous system. The former produced depression with impairment of motor activity, while the latter caused excitation and clonic convulsions. Both were shown to act on the brain stem with either compound capable of antagonizing the other.

In a study by Maffii (3), it was reported that 3-phenyl and 3-phenyl-5-hydroxy-1,2,4-triazole had paralytic effects and exerted protection against the tonic phase of maximal electroshock and antagonized the convulsive and lethal effects of strychnine but not of metrazole.

Polya (4) reported that, while working with water-soluble 1,2,4-triazoles during the determination of cholinesterase inhibition activity, laboratory workers developed light symptoms of nicotine or physostigmine poisoning. 3,5-Dimethyl-1-phenyl, 3-ethyl-5-methyl-1-phenyl, and 3-methyl-5-ethyl-1-phenyl-1,2,4-triazole were found, in concentrations between 0.01 and 0.5 *M*, to inhibit cholinesterase activity.

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EXPERIMENTAL

Since some phenyl triazoles were shown to have central nervous system effects and others to produce cholinesterase inhibition, 3,5-DPT was checked for such activities. At the same time other pharmacological actions were studied. Several species of animals were used in these studies, including Harlan strain albino mice, Wistar strain albino rats, *Rana pipiens* frogs, and cats. The triazole for administration was prepared in two forms. Solutions of 0.01 to 5.0% were prepared by gently warming the compound in concentrated sodium hydroxide solution and neutralizing with hydrochloric acid to a pH of 9.8. Suspensions of 1.0 to 2.0% were prepared by triturating one part of 3,5-DPT to two parts of acacia, with the gradual addition of water, in a mortar.

Median Lethal Doses.—3,5-DPT was administered i. p. and i. v. as 1.0 to 2.0% solutions to albino mice of mixed sexes weighing 14 to 24 Gm. to determine the median lethal dose (LD_{50}). A dose probit curve for each route was obtained from which the LD_{50} and its standard error (S. E.) were computed by the Miller-Tainter method (5). The doses that were lethal produced death within the first two hours after intravenous injection with convulsions and anoxia the apparent causes of death. Death which ensued within the first twenty-four hours after intraperitoneal injection was apparently due to respiratory depression. The intraperitoneal LD_{50} was 300.0 ± 42.8 mg./Kg. and the intravenous LD_{50} was 89.5 ± 10.2 mg./Kg.

Behavior Studies.—Albino mice of mixed sexes weighing 15 to 25 Gm. and frogs (*Rana pipiens*) weighing 50 to 60 Gm. were utilized for these studies. 3,5-DPT in doses of 50, 100, and 200 mg./Kg. was administered intraperitoneally to the mice, and the animals were observed and compared with normal untreated animals for a period of two hours. Doses of 2.5, 5.0, 10.0, 20.0, and 50.0 mg. of 3,5-DPT in acacia suspensions were injected into the ventral lymph sac of the frogs, and these animals were compared with normal untreated subjects for a period of four hours.

In mice, doses of 100.0 and 200.0 mg./Kg. of 3,5-DPT caused an increase in activity and irritability, followed within ten to fifteen minutes by a marked decrease. Respiration was depressed at 50.0 mg./Kg. following an initial increase. Only depression was observed with the higher doses (100.0 and 200.0 mg./Kg.). Marked escape and placing loss were observed at all dose levels with grasping loss, stay center, arching, and rolling. In frogs, all doses except 50 mg. produced spontaneous hyperactivity and mild convulsive states which appeared within fifteen minutes and lasted for forty-five minutes. The animals remained hypersensitive for the duration of the observational period. Convulsions which ensued due to activating the animal were characterized by a reverse-C curve, crossing of the forelegs under the chin, and muscular rigidity. At 50.0 mg., only paralysis and rigidity were observed.

Effect Upon Barbiturate Sleeping Time.—Prolongation of hexobarbital sleeping time is a useful procedure to detect depression that may be inadequate to produce readily observable symptoms. Since 3,5-DPT exhibited characteristics of both depression and stimulation in the behavioral studies, the effect of 3,5-DPT on barbiturate activity was determined. For this study forty albino mice weighing 16 to 18 Gm. were divided into four groups of ten animals each. The triazole was administered intraperitoneally prior to the barbiturate. Groups I, II, and III received 30.0, 100.0, and 150.0 mg./Kg., respectively, as a 0.5% solution. Fifty minutes later all four groups received 100.0 mg./Kg. of hexobarbital. Group IV receiving only hexobarbital was retained as the control group.

All animals following this treatment were sufficiently depressed to permit placing them on their backs. The sleeping time of each animal was then measured as the time which elapsed between injection of the barbiturate and return of the righting reflex. Each sleeping time was converted to its logarithm, and the geometric mean and standard error calculated for each group. The results which show the prolongation by 3,5-DPT at all except the 30.0 mg./Kg. level, are given in Table I.

Effect upon Maximal Electrical Convulsive Threshold.—The method employed for this study was the method reported by Toman, Swinyard, and Goodman (6). The apparatus required was an electrical stimulator described by Woodbury and Davenport (7). Rats weighing between 110 and 135 Gm. were selected as test animals. A prescreened colony of animals was used consisting only of animals which exhibited tonic extension when subjected to a current of 150 ma. for 0.2 seconds delivered by ear clip electrodes.

This experiment was performed by dividing the animals into six groups of four animals each. All animals received the drug by intraperitoneal route with groups I and IV receiving 150 mg./Kg., groups II and V receiving 100 mg./Kg. of 3,5-DPT, and groups III and VI receiving equivalent amounts of alkaline vehicle control. The experiment was designed so that alternate groups received the electroshock at alternate hourly periods so as to reduce the possibility of changing the convulsive threshold. An animal was considered protected if he no longer exhibited the tonic extensor component of the clonic-tonic convulsion. The shocking procedure was continued until two consecutive hourly shocks showed no remaining protection.

3,5-DPT was found to protect 75% of the animals against electroshock convulsions at 150 mg./Kg.

Table I.—3,5-DPT Effect Upon Duration of Hexobarbital Sleep

Drug	*Dose, mg./Kg.*[a]	*Geom. Mean Sleeping Time ± S. E., min.*	*Per Cent Change from Control ± S. E.*
3,5-DPT	30.0	36.06 ± 1.10	(−) 7.57 ± 0.10
3,5-DPT	100.0	46.46 ± 1.14	(+) 19.09 ± 0.71
3,5-DPT	150.0	75.34 ± 1.24	(+) 93.13 ± 3.07
Control (hexobarbital only)		39.01 ± 1.11	

[a] Administered intraperitoneally.

for a duration of two hours, and 50% at 100 mg./Kg. for a duration of an hour. Therefore, 3,5-DPT can be said to exert only brief protection and only at high doses against electrically induced convulsions.

Effect upon Metrazole-Induced Convulsions.—Although only slight activity of 3,5-DPT was observed in the electroshock procedure, the effect upon metrazole-induced convulsions was determined. Certain drugs are known to act against chemically induced seizures, while others act against electroshock seizures. For this study albino mice weighing between 18 and 23 Gm. were used. Six mice were injected intraperitoneally with 150 mg./Kg. of 1% solution of 3.5-DPT. Thirty minutes later 100 mg./Kg. of metrazole was injected by the same route into the animals. Any animal that did not exhibit convulsions of five-seconds duration within thirty minutes after metrazole injection was considered protected. No protection was seen with 150 mg./Kg. of 3,5-DPT against chemically induced metrazole seizures.

Determination of Analgetic Action.—Since analgesia was observed at high doses the possibility of analgesia at lower doses accompanying the observed depression was not overlooked. The method used was essentially the same as the method reported by Davies and co-workers (8). For this study mice weighing 16 to 18 Gm. were used. One each was injected at doses of 50, 70, 100, 140, and 200 mg./Kg. Normal reaction time was determined prior to injection of 0.5% solution of drug. At twenty minutes, reaction time was again determined and per cent change in reaction time was calculated.

The results of this experiment showed no significant analgesia occurring after injection of 3,5-DPT. Pain threshold was lowered very slightly at all levels except the near lethal 200 mg./Kg. dose which elevated the threshold 71%. However, due to the marked ataxia observed, the action could not be attributed to analgesia.

Cholinesterase Inhibition Activity.—Although no detectable symptoms of autonomic activity were observed in behavior studies, the possibility of peripheral parasympathetic action was not discarded. A procedure was followed to determine the specific cholinesterase inhibition of blood serum. Albino rats of mixed sexes weighing 225 to 345 Gm. were used and divided into three groups of five animals each. The groups were injected as follows: I, 100 mg./Kg. 3,5-DPT (2% suspension); II, 0.6 mg./Kg. physostigmine sulfate (0.02% solution); III, water control.

One hour after drug administration, the animals were sacrificed and bled from the external jugular vein. The blood samples obained were centrifuged. One milliliter of the supernatant serum of each sample was diluted with 9 ml. of water. The pH of each tube was adjusted to a slightly basic end point with 0.01 *N* sodium hydroxide, using two drops of thymolphthalein indicator. One milliliter of 5% methacholine solution was added to each tube, followed by a twenty-minute period of incubation in a 38° water bath.

The acetic acid liberated was titrated with 0.01 *N* sodium hydroxide. The control tubes (group III) were considered as having exhibited normal cholinesterase activity. The degree of inhibition in the other samples was calculated on the difference in ability to hydrolyze methacholine. The results given in Table II show that 3,5-DPT inhibits cholinesterase to a minor degree as compared to physostigmine.

Blood Pressure, Vagal and Flexor Reflex Stimulation in Cats.—These studies were made to investigate the systemic actions which could not be adequately evaluated on smaller animals. All animals were anesthetized with 35 mg./Kg. of secobarbital except those that were to be used to test drug action upon reflex arc. These animals were given methohexital intravenously initially followed by 50 mg./Kg. of chloralose in a polyethylene glycol vehicle, administered through polyethylene tubing into the saphenous vein.

The trachea was cannulated, and both carotid arteries and the right vagus nerve were isolated. The right posterior tibial nerve and the tendinous insertion of the right anterior tibialis muscle were exposed and dissected free of surrounding tissue. The muscular branch of the right sciatic and the right femoral nerves were isolated and severed. The right femur was exposed midway between its articulations and a clamp affixed to immobilize the right leg. Blood pressure was recorded from the left femoral artery and respiration was recorded from the tracheal cannula in the usual fashion.

Right nictitating membrane response was recorded by a pulley attached to the membrane by a string. Intestinal response was recorded either by pulleys from a loop of the tract or by a balloon inserted into the tract and connected to a tambour. Right anterior tibialis muscle response was recorded by a weighted pulley which was attached to the tendon of the muscle by a thread. Muscle contraction was caused by stimulation of the right posterior tibial nerve to which was attached shielded electrodes delivering sufficient voltage to cause well-defined, reproducible response and at a frequency of one per second for a duration of thirty seconds. Vagus stimulation was produced by sufficient voltage at one impulse per second for ten seconds. Carotid occlusion was effected by clamping off both carotid arteries for thirty seconds.

Typical responses were routinely produced in these animals by epinephrine, methacholine, and histamine. These were administered before and after

TABLE II.—CHOLINESTERASE INHIBITION IN RATS

Drug	Dose, mg./Kg.	Group	Ml. 0.01 N NaOH to Titrate ± S. E.	% Inhibition ± S. E.
3,5-DPT	100.0	I	3.43 ± 0.24	36.95 ± 2.64
Physostigmine sulfate	0.6	II	2.57 ± 0.08	52.76 ± 1.82
Control		III	5.44 ± 0.06	

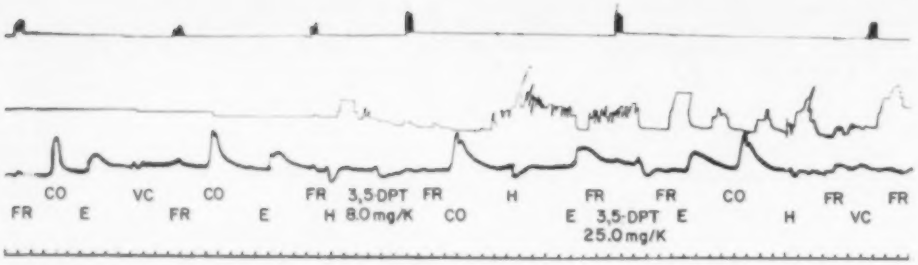

Fig. 1.—Female cat, 1.7 Kg., chloralose anesthesia. Top line is the contraction of the anterior tibialis muscle. Second line is the balloon intestinal response. Third line is blood pressure. Bottom line is the time in minutes. *FR*, stimulation of the anterior tibialis muscle; *CO*, carotid occlusion; *E*, epinephrine 0.0025 mg./Kg.; 3,5-DPT, 3,5-diphenyl-1,2,4-triazole; *H*, histamine 0.005 mg./Kg.

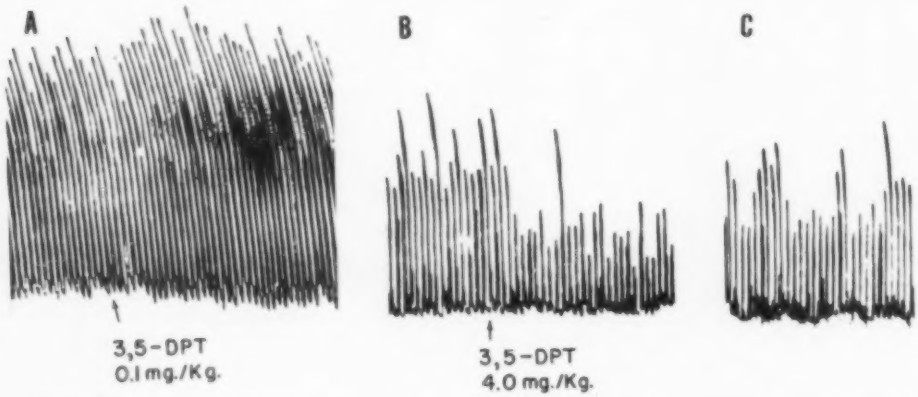

Fig. 2.—Rat gastrocnemius-sciatic preparation, intact. The tracing shows contractions of the left gastrocnemius-muscle in response to stimuli applied to the left sciatic nerve at five-second intervals. *A*, 3,5-DPT (3,5-diphenyl-1,2,4-triazole) 0.1 mg./Kg.; *B*, 3,5-DPT 4.0 mg./Kg.; *C*, recovery after a rest of twenty minutes.

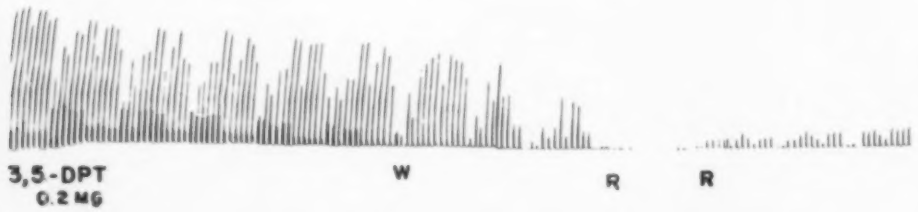

Fig. 3.—Isolated frog gastrocnemius-sciatic preparation. The tracing shows the contractions of the right gastrocnemius muscle in response to the right sciatic nerve stimulation at five-second intervals. 3,5-DPT, 3,5-diphenyl-1,2,4-triazole, 0.20 mg.; *W*, wash; *R*, rest fifteen minutes.

triazole administration to determine possible 3,5-DPT influence upon their effects. Representative tracings from such studies are shown in Fig. 1.

With administration of low doses of 3,5-DPT, blood pressure was increased and then decreased. At medium and high doses pronounced hypotension was observed. Relaxation of the intestine occurred at all but very high dose levels where the resulting contraction was relaxed by administration of epinephrine. 3,5-DPT produced little effect upon respiration.

It was found that 3,5-DPT caused slight enhancement of carotid occlusion and epinephrine and methacholine responses. Occasionally, at high doses a decrease in epinephrine and methacholine responses was observed. At medium and high

doses 3,5-DPT caused a decreased response to occur upon stimulation of the vagus. A consistent decrease in histamine activity was evident.

The flexor reflex was the response affected most by triazole administration. A marked increase in response was observed at low and medium doses, while high doses produced a complete block of the reflex. It was possible to elicit response by direct muscle stimulation during the block which was found to be reversible in character.

3,5-DPT Action in Spinal Cats.—Cats with high cervical spinal cord transection were used to determine whether the effects observed in the anesthetized cats necessitated the presence of the higher centers in the central nervous system. Animal preparations were identical to those of the anesthetized cats, with the exception of cord transection at the second cervical vertebra which necessitated the use of artificial respiration. Primary interest in this preparation was the effect of 3,5-DPT upon the flexor reflex. Blockage and recovery of the reflex occurred sooner than in the nonspinal animals. Increased stimulation initially at low and medium doses was not as pronounced as in the anesthetized animals. Epinephrine, methacholine, and histamine responses were essentially the same with enhancement of the former two and decrease in response of the latter.

Intact Nerve-Muscle Preparation.—The action of 3,5-DPT on the flexor reflex in both the anesthetized and spinal cat brought an increased interest in possible action at the myoneural junction. For this study the gastrocnemius-sciatic preparation in intact albino rats weighing 250 to 350 Gm. was used. The test animals were anesthetized with 180 mg./Kg. of phenobarbital administered intraperitoneally. The external jugular vein was cannulated with polyethylene tubing through which the drug was administered. The sciatic nerve on the right side was isolated and shielded electrodes were attached distally to a ligature. The right gastrocnemius muscle was isolated and attached by means of pulleys to a recording lever. The muscle contractions produced by sufficient voltage delivered to the sciatic nerve every five seconds were recorded on a smoked kymograph. Representative tracings may be seen in Fig. 2 and show a slight increase in contraction due to the administration of low doses of 3,5-DPT with a partial reversible blockage occurring at higher doses. Vehicle controls were run and found to be devoid of interference or action.

Isolated Nerve-Muscle Preparations.—Supportive studies were run on another species with frogs (*Rana pipiens*) weighing 50 to 60 Gm. The sciatic nerve and the gastrocnemius muscle were isolated and removed. The muscle was inserted in a muscle bath of cold blooded Ringer's solution and attached to a recording lever. The muscle contractions produced by stimulation of the sciatic nerve with sufficient voltage every five seconds were recorded on a smoked kymograph. Representative tracings seen in Fig. 3 show the occurrence of a complete and reversible block. Vehicle controls were found to be insignificant in producing activity on this preparation.

DISCUSSION

The experimental evaluation of the pharmacological action of 3,5-diphenyl-1,2,4-triazole shows it to have multiple activity of central stimulation and depression and cholinergic activity characteristic of cholinesterase inhibition. The latter effect was assumed to be separable from activity caused by the central autonomic effects since flexor reflex enhancement followed by blockage persisted in the spinal animal. Vagal stimulation, flexor reflex, and carotid occlusion indicate that the ganglionic components of the cholinergic activity are prominent. Other isolated and nerve-muscle preparations show that the skeletal muscle component is also present.

The weak activity against electroshock and barbiturate prolongation indicates that the central depression probably does not originate primarily from the higher portions of the cerebrospinal axis. Neither may the medulla be regarded as the predominant locus of action as pronounced blood pressure and respiration changes are seen only with very high doses. Thus, it appears that other areas of the cerebrospinal axis are the primary site of depression caused by the drug.

The site of the central stimulatory activity seen could not be pinpointed on the basis of these studies. Supportive enzymatic determinations of amine oxidase inhibition might greatly contribute to the further elucidation of the pharmacological activity of 3,5-diphenyl-1,2,4-triazole.

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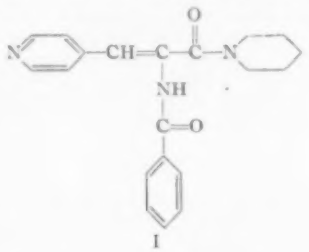
Pharmacological Studies on Some New Azlactone Derivatives

By G. ALAN ROBISON and F. W. SCHUELER

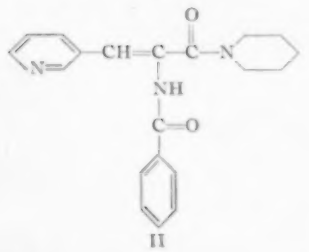
Several members of a new series of azlactone derivatives (substituted acrylic acid amides), which by themselves exhibit convulsant properties, were found to be capable of prolonging the duration of hexobarbital-induced hypnosis in mice. Experiments are described which indicate that this action is secondary to inhibition of the biotransformation of hexobarbital in the liver. One member of the series was found to be a long-acting depressant capable of potentiating the action of strychnine.

THE CHEMICAL and physical properties of the azlactones [5(4)-oxazolones] have been well studied (1–4), but there have been relatively few investigations of the pharmacological activity of either the azlactones themselves or (with the exception of the α-amino acids) their many possible derivatives. Schueler and Hanna (5) studied the cardiac activity of a series of azlactones and related products, and Cronheim, *et al.* (6), recently reported on the interesting sedative properties of trimethoxybenzoyl-glycine-diethylamide, which could be thought of as the derivative of a saturated azlactone (4).

In testing several new series of these derivatives for pharmacological activity, our attention was initially drawn to a series of amides. Of particular interest was the finding that α-benzoyl-amino-β-(4-pyridyl) acrylic acid piperidide (I) was a central stimulant, whereas its 3-pyridyl congener (II) acted to depress the CNS. This relationship does not exist in the case of their respective diethylamide analogues, which are both convulsants.



I



II

On studying the central activity of these agents further, the discovery was made that I (and both the diethylamides) would, despite their convulsant properties, markedly prolong the duration of hexobarbital-induced hypnosis in mice, whereas II was relatively inactive in this respect.

Studies were subsequently initiated to determine the mechanism of action of this effect. The results of these studies, together with the results of studies designed to characterize the nature of the central activity of the compounds, are reported herein.

EXPERIMENTAL

Preparation of Materials.—The azlactones used as intermediates were prepared by reacting the appropriate acyl glycine with the appropriate aldehyde in the presence of acetic anhydride and sodium acetate, as previously described (2), although heating is not required in the case of the pyridinecarboxaldehydes. Commercially unavailable acyl glycines were prepared by the regular Schotten-Baumann reaction. The majority of the azlactones were recrystallized from 95% ethanol.

The amides were prepared by the method B of Barnes, *et al.* (1), and were recrystallized from benzene-hexane. Those amides not previously reported in the literature are shown in Table I. The yields varied from about 40 to 80%.

Preliminary Screening.—The compounds were administered to Swiss albino mice in doses up to at least 500 mg./Kg. (orally, in suspension with 0.5% carboxymethylcellulose), and the gross effects observed. With reference to Table I, compounds 5 to 10 were administered parenterally. LD_{50}'s were determined for some of the compounds (for mice by i. p. injection), either by a simplified method of probits (7) or by the method of moving averages (8). Rectal temperatures (mice) were measured by means of a YSI model 43 tele-thermometer.

Drugs used in attempting to antagonize the convulsant effect of I (compound No. 5 in Table I)

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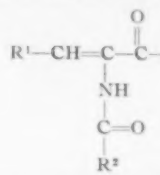
TABLE I.—SUBSTITUTED ACRYLIC ACID AMIDES



No.	R^1	R^2	NR_2	M. P., °C.[a]	Formula		Analyses[b] C, %	H, %	N, %
1	Phenyl	tmp[c]	Piperidino	decompn. 180	$C_{24}H_{28}N_2O_5$	Calcd.	67.90	6.60	6.60
						Found	68.31	6.81	6.50
2	tmp	tmp	Piperidino	decompn. 198	$C_{27}H_{34}N_2O_8$	Calcd.	63.00	6.60	5.44
						Found	62.73	6.55	5.79
3	tmp	Phenyl	Piperidino	160–162	$C_{24}H_{28}N_2O_5$	Calcd.	67.90	6.60	6.60
						Found	66.94	6.40	6.53
4	tmp	Phenyl	Diethylamino	196–197	$C_{22}H_{26}N_2O_5$	Calcd.	67.00	6.80	6.80
						Found	66.27	6.52	7.03
5	4-Pyridyl	Phenyl	Piperidino	195–196	$C_{20}H_{21}N_3O_2$	Calcd.	71.65	6.26	12.50
						Found	71.25	6.11	12.29
6	4-Pyridyl	Phenyl	Diethylamino	197–198	$C_{19}H_{21}N_3O_2$	Calcd.	70.50	6.50	13.00
						Found	69.83	6.46	12.56
7	4-Pyridyl	Methyl	Piperidino	148–149	$C_{15}H_{19}N_3O_2$	Calcd.	66.00	6.95	15.39
						Found	66.18	6.88	15.61
8	4-Pyridyl	tmp	Piperidino	decompn. 169	$C_{23}H_{27}N_3O_5$	Calcd.	65.00	6.35	9.88
						Found	65.48	6.57	9.69
9	3-Pyridyl	Phenyl	Piperidino	187–189	$C_{20}H_{21}N_3O_2$	Calcd.	71.65	6.26	12.50
						Found	71.61	6.26	12.73
10	4-Pyridyl	Phenyl	Diethylamino	186–187	$C_{19}H_{21}N_3O_2$	Calcd.	70.50	6.50	13.00
						Found	70.62	6.50	13.03

[a] Melting points are uncorrected. [b] Analyses are by Alfred Bernhardt, Max Planck Institute, Mulheim, W. Germany. [c] tmp = 3,4,5-Trimethoxyphenyl.

included pyridoxine HCl, hexobarbital, zoxazolamine, and trimethadione. Drugs used in combination with II (compound No. 9 in Table I) included, in addition to its 4-pyridyl congener (I), strychnine and pentylenetetrazol.

Hexobarbital Sleeping Time.—This was measured as the duration of loss of righting reflex, the mice being considered asleep until they could right themselves three times in one minute. Controls were always run along with the experimental group(s), and the significance of the difference between the mean sleeping times of the control and experimental group(s) was determined by means of Student's "*t*" test. Compound No. 5 (I) was used as an additional control in all experiments. The various amides used to pretreat the mice were either given orally, in suspension with 0.5% carboxymethylcellulose, or, when only the pyridyl derivatives were being compared, parenterally (s. c. or i. p.) as the hydrochloride or tartrate salts. Hexobarbital was always administered intraperitoneally as the sodium salt, using a dose of either 110 mg./Kg. or 150 mg./Kg. Results are expressed in terms of per cent prolongation of the mean control sleeping time.

Hexobarbital Metabolism.—For the determination of the effect of compound No. 5 on total body hexobarbital concentration, the experimental mice were pretreated with 25 mg./Kg. of the drug, subcutaneously, followed in thirty minutes by the intraperitoneal administration of hexobarbital (200 mg./Kg). One hour after the administration of the hexobarbital, the total body concentration (mg./Kg.) was determined, either by the method of Brodie, *et al.* (9, 10), or by the one used by Fujimoto, *et al.* (11). Results by either method are in close agreement.

Isolated rat liver perfusions were performed to localize the effect of Tu-702 on hexobarbital metabolism at the organ level. The livers were obtained from male Sprague-Dawley or Long-Evans rats weighing 400–550 Gm. They were perfused (100 ml. albumin-Locke solution plus 50 ml. freshly drawn whole rat blood) in a recirculating system similar to that of Plaa and Hine (12). The perfused liver was given approximately one hour to equilibrate with its new environment and then the disappearance of added hexobarbital from the system was studied in the presence and absence of compound No. 5. The rate of disappearance of added hexobarbital from the system was followed by determining the hexobarbital concentration in aliquots of the perfusate taken at ten, twenty, forty, eighty, and one hundred and twenty minutes, and by determination of the terminal concentration of hexobarbital in the liver at one hundred and twenty minutes.[1]

RESULTS

Of the compounds listed in Table I only those having a pyridyl moiety attached to the β-carbon caused grossly observable effects in mice (in doses up to 500 mg./Kg). Compounds 5, 6, and 10 were convulsants, superficially resembling pentylenetetra-

[1] The authors wish to express their gratitude to Dr. Gabriel L. Plaa for his invaluable assistance in carrying out the liver perfusions.

zol in this respect. Of the anticonvulsants tested, trimethadione was by far the most effective (Table II). Zoxazolamine and hexobarbital were less effective in that they protected only after doses that caused obvious depression. Pyridoxine was completely ineffective.

The depressant effect of compound 9, the only member of the series that was found to have this effect, was of long duration. In doses less than those required to cause loss of the righting reflex, its most prominent effect in mice was the production of a state of catalepsy. After a dose of 400 mg./Kg. the mice would in many cases "sleep" (lose their righting reflex) for longer than twelve hours. A surprising observation was that pretreatment with this compound lowered the threshold to convulsions induced by either its 4-pyridyl congener (compound 5) or strychnine, as shown in Table III. Pentylenetetrazol, on the other hand, was not potentiated by the compound.

Neither the dose of the 4-pyridyl congener (compound 5) nor of the strychnine sulfate were sufficient to induce convulsions in the control mice, but they became very potent convulsant doses after pretreatment with the depressant. An interesting observation was that after pretreatment with doses sufficient to cause loss of the righting reflex, the subsequent administration of 1.5 mg./Kg. of strychnine sulfate would not necessarily induce convulsions, but was invariably lethal (death being due to respiratory failure). Like its depressant effect, the ability of the depressant to potentiate the action of its 4-pyridyl congener and of strychnine was of long duration.

Table II.—Protection by Trimethadione Against Convulsions Induced by α-Benzoylamino-β-(4-pyridyl) Acrylic Acid Piperidide[a]

	Ratio of Number Convulsing to Number Treated	Ratio of Number Dying to Number Treated
Control (no pretreatment)	10/10	9/10
Pretreated with trimethadione, 500 mg./Kg. (i. p.), 15 min. before convulsant	0/10	0/10

[a] Swiss albino mice, 100 mg./Kg., s. c. injection.

Table III.—Effect of Pretreatment with α-Benzoylamino-β-(3-pyridyl) Acrylic Acid Piperidide[a] on Lethality of Various Convulsants

	Ratio of Number of Mice Dying to Number Treated with the Corresponding Convulsant	
	Controls (no Pretreatment)	Pretreated
Compound 5 (Table I), 50 mg./Kg., i. p.	0/10	10/10
Strychnine sulfate, 1.5 mg./Kg., i. p.	0/10	10/10
Pentylenetetrazol, 100 mg./Kg., i. p.	9/10	4/10

[a] A 200 mg./Kg. dose, s. c., thirty minutes before administration of the convulsant.

The effect on the duration of hexobarbital-induced hypnosis in mice varied depending on the conditions of the experiment but, using a dose of hexobarbital of 110 mg./Kg. and a pretreatment time of forty minutes, 50 mg./Kg. of the 4-pyridyl piperidide caused a prolongation in sleeping time of 996% (i. e., 24.1 ± 0.9 minutes for the controls *vs.* 263.8 ± 17.5 minutes after pretreatment). Taking the per cent prolongation after the 4-pyridyl piperidide in each experiment as unity, the relative potencies of the other derivatives tested are listed in Table IV. Also shown in this Table is the significance of the difference between the effect of each of the compounds and that of the 4-pyridyl piperidide. Shown, in addition, is the estimated LD_{50} for each of the compounds (based on 24 to 48 mice per assay).

Table IV.—Prolongation of Hexobarbital-Induced Hypnosis in Mice

No. (from Table I)	Potency Relative to Compound 5[a]	P	LD_{50}, mg./Kg.
5	1.00	..	74
6	1.06	>0.05	132
7	0.43	<0.05	>1600
8	0.52	<0.05	>800
9	0.19	<0.01	476
10	0.89	>0.05	126

[a] Doses equimolar to doses used of compound 5.

In all cases the dose used of each of the derivatives was equimolar with the dose used of the 4-pyridyl piperidide. Under these conditions the compounds listed in Table I but not shown in Table IV caused no significant prolongation of the control sleeping time.

Table V shows the effect of compound 5 on hexobarbital body levels in mice. It will be seen from these data that in one hour the rate of disappearance is reduced by almost one-half by pretreatment with this drug.

Table V.—Effect of α-Benzoylamino-(4-pyridyl) Acrylic Acid Piperidide on Hexobarbital Body Levels

	Hexobarbital, mg./Kg. Initial	Hexobarbital, mg./Kg. After 60 min.[a]	Disappearance, %
Controls (6 mice)	200	80.3 ± 3.5	59.9
Pretreated (6 mice)	200	133.7 ± 15.3	33.2

[a] ± Standard error.

The results of the liver perfusion experiments are shown by means of decay curves (Fig. 1), hexobarbital concentration being expressed as a percentage of the concentration initially present (approx. 650 mcg./ml.). While these curves each represent the results of a single experiment, the results of duplicate experiments were in excellent agreement with those illustrated. The control curves were calculated to follow most closely a second-order rate reaction, with a half-life (based on the initial concentration) of about forty minutes.

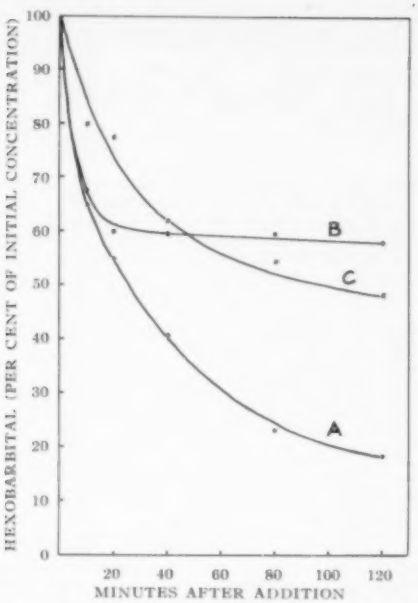


Fig. 1.—Effect of α-benzoylamino-β-(4-pyridyl) acrylic acid amide (I) on rate of disappearance of hexobarbital from rat liver perfusate. *A*, control; *B*, 25 mg. of I added fifteen minutes before hexobarbital; *C*, 25 mg. I added thirty minutes before hexobarbital.

DISCUSSION

The relationship between structure and activity in this series of compounds represents something of a paradox. The situation in the case of the structures (i. e., I being a convulsant and II a depressant) does not exist in the case of their diethylamide analogues, the transition from diethylamino to piperidino having in fact a different effect depending on the position of the nitrogen in the pyridyl ring. In the 4-pyridyl series this change, represented by the transition from compound 6 to compound 5, results in a quantitative change only (an increase in toxicity, as shown by the LD_{50}'s in Table IV). The same change in the 3-pyridyl series, represented by the transition from compound 10 to compound 9, corresponds to an opposite change in acute toxicity and, in addition, there is a qualitative change from stimulant to depressant.

Based on gross observations, the convulsants in this series appear to be of the same type as pentylenetetrazol, a suggestion supported by the antagonistic effect of trimethadione (13). Pentylenetetrazol has a stimulating effect on excitatory synapses within the central nervous system (14).

The depressant effect of α-benzoylamino-β-(3-pyridyl) acrylic acid piperidide is poorly understood, but its ability to potentiate strychnine is interesting. Unlike pentylenetetrazol, strychnine is a convulsant by virtue of its ability to depress inhibitory synapses within the CNS (14, 15). One class of depressants that potentiate strychnine are the erythrina alkaloids, which are capable of blocking transmission between motor-axon collaterals and Renshaw cells (16). Whether a similar mechanism of action can be invoked in the case of the 3-pyridyl piperidide remains to be seen. The fact that after pretreatment with this compound strychnine is lethal without necessarily causing convulsions suggests that strychnine's ability to inhibit transmission at excitatory synapses (15) may be of importance in explaining some of its gross effects.

Substitution of either methyl or trimethoxyphenyl (as in compounds 7 and 8, respectively) for the phenyl moiety of compound 5 results in a drastic reduction in central activity, although the ability to prolong the duration of action of hexobarbital is retained to a considerable degree, as shown by the data in Table IV. The prolongation of hexobarbital sleeping time is probably secondary to inhibition of the biotransformation of hexobarbital in the liver, which is the principal means by which the action of hexobarbital in the body is terminated (17).

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Steroid Borates II. Process Applications

By LEWIS J. LEESON, JAMES A. LOWERY, GEORGE M. SIEGER, and SIEGFRIED MULLER

Alkaline steroid borates, in addition to exhibiting high aqueous solubility, have been found useful in certain chemical processes. These include separation of 16α,17α-hydroxylated steroids from steroids lacking the *cis* glycol moiety at C_{16} and C_{17}, and the preparation of 21-monoesters of 16α-hydroxylated adrenocorticoids. Both of these techniques are based upon the complexing of 16α,17α-dihydroxy steroids with alkaline borates.

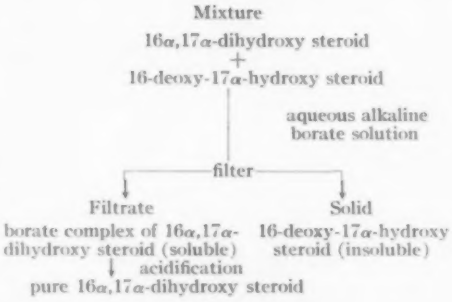
IN A PREVIOUS publication (1) a method of preparing water-soluble borates of 16α,17α-hydroxylated adrenocortical steroid hormones was presented. Three important characteristics of these materials are (*a*) ease of formation, (*b*) high water solubility, and (*c*) their ready decomposition to the parent steroid by mild acidification. These phenomena allow for application of the steroid borates in various separation and purification techniques, two of which are discussed in this paper.

The high aqueous solubility demonstrated by the alkaline steroid borates may also prove useful in pharmaceutical formulation work. The incorporation of these materials into various dosage forms is presently under investigation.

EXPERIMENTAL

Separation of 16α,17α-dihydroxy Steroids from 16-Deoxy-17α-hydroxy Steroids.[1]—Early experiments indicated that while alkaline borate solutions were capable of conferring high water solubility upon 16α,17α-dihydroxy steroids, such as triamcinolone,[2] 9α - fluoro - 16α - hydroxyhydrocortisone, 16α - hydroxy prednisolone, etc., the same alkaline borate solutions did not influence the solubility properties of steroids in which the C_{16}, C_{17} *cis* glycol function was absent. Since, in the synthesis of triamcinolone, the separation of 16α,17α-dihydroxy type steroids from their 16-deoxy precursors is of great commercial importance, it was felt that the phenomena of selective solubilization by alkaline borates might be utilized for this purpose. The proposed procedure is outlined qualitatively in scheme A.

In order to test the proposed separation principle several two-compound mixtures were prepared each containing a 16α,17α-dihydroxy steroid and a 16-deoxy-17α-hydroxy steroid. Each of the mixtures, prepared by physically mixing the dry solids, was added to an alkaline borate solution and allowed to equilibrate at room temperature overnight with stirring. The undissolved solids were removed by filtration, washed well with water, and dried. In every instance the infrared spectra of the undissolved solids were identical with those of the 16-deoxy-17α-hydroxy components. The clear alkaline borate solutions (filtrates) upon acidification to below pH 2 with dilute hydrochloric acid deposited solids which were collected, washed, and dried. In every instance the infrared spectra of these solids were identical with those of the pure 16α,17α-dihydroxy steroid components of the mixtures. The results of these experiments are summarized in Table I and the accompanying identifying structures.



Scheme A

Having thus verified the fundamental separation principle, it remained necessary to apply the separation technique to the crude product obtained from the microbiological oxidation of 9α-fluorohydrocortisone. The following procedure was found to work satisfactorily. Eight liters of mash from a ninety-hour fermentation of 9α-fluoro-hydrocortisone (II) with *Streptomyces roseochromogenus* (2) was extracted with methyl isobutylketone. A 2-L. aliquot of this extract [containing 9α-fluoro hydro-

TABLE I

Mixture	Infrared Spectrum: Undissolved Solid	Infrared Spectrum: Acid Precipitated Solid
I and VI	I	VI
I and V	I	V
III and VI	III	VI
IV and VI	IV	VI

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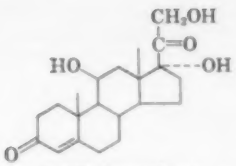
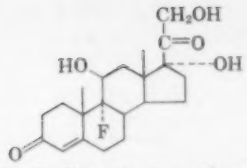
The authors are indebted to Mr. William Fulmor and his staff for their help in interpreting the infrared curves mentioned herein.

[1] The presence of both the 16α and 17α hydroxyl groups is necessary for formation of the borate complex because of the nature of the chemical bonds involved (1). In practice, any steroid without the 16α,17α-dihydroxy moiety will not combine with alkaline borates; the 16-deoxy-17α-hydroxy steroids are used as examples (rather than the alternate possibilities), because these types are the most common ones encountered.

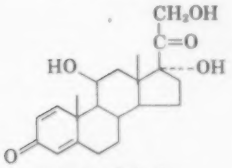
[2] American Cyanamid Co.'s trademark for triamcinolone is Aristocort.



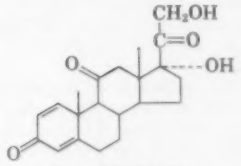
I Hydrocortisone



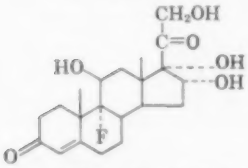
II 9α-Fluoro-hydrocortisone



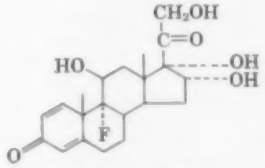
III Prednisolone



IV Prednisone

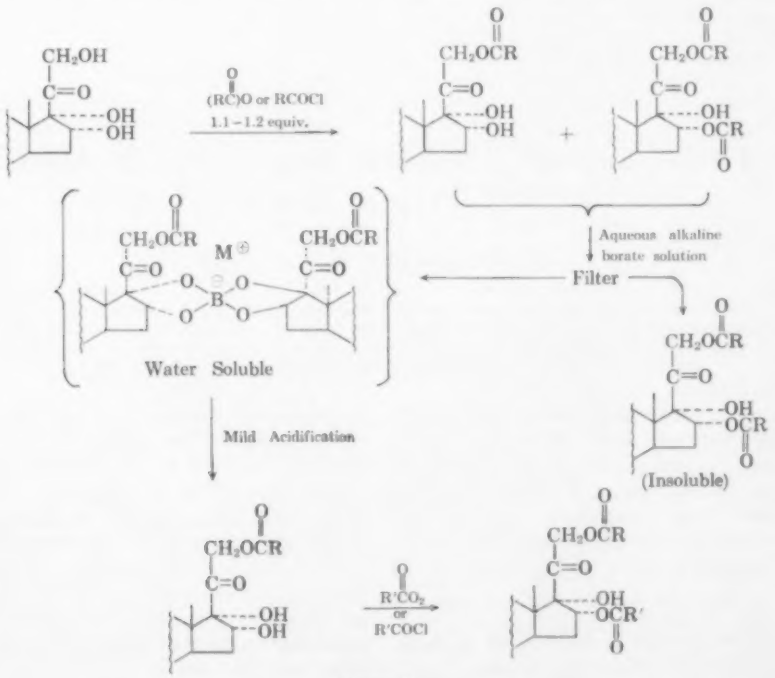


V 9α-Fluoro-16α-hydroxyhydrocortisone



VI Triamcinolone

cortisone (II) and 9α-fluoro-16α-hydroxy-hydrocortisone (V)] was further extracted with five 20-ml. portions of 0.1 *M* aqueous sodium tetraborate solution. The aqueous extracts were combined, filtered, and adjusted to pH 1.5–2.0 with concentrated hydrochloric acid. After standing overnight at room temperature, crystals of 9α-fluoro-16α-hydroxy hydrocortisone which had formed were collected by filtration. Hydrolysis of any 9α-fluoro-16α-hydroxy hydrocortisone borate (free acid) present in the solid product is accomplished by adding 25 ml. of methanol and evaporating to dry-



Scheme B

ness. This procedure was repeated several times during which methyl borate was formed by transesterification and removed by evaporation (b.p. 65°).

The 16α,17α-dihydroxy steroid (V) recovered using the above procedure was found to have a purity better than 98% according to blue tetrazolium-spectrophotometric (3) and polarographic assays. This separation procedure constitutes a marked improvement over current techniques which involve separation and purification of the 16α,17α-dihydroxy steroids via chromatography and/or fractional crystallization.

Use of the Borate Separation Principle in the Preparation of C_{21}-Monoesters and C_{16},C_{21}-mixed Esters of Triamcinolone and Related 16α-Hydroxylated Corticosteroids.—The treatment of 16α-hydroxylated steroids with acylating reagents, even under carefully controlled reaction conditions, invariably results in complex mixtures. For example, triamcinolone (VI), upon reaction with one equivalent of an acid anhydride or acid halide, gives a mixture which contains 16,21-diester and 21-monoester as well as unreacted triamcinolone. A 10 to 20% excess of the acylating reagent provides a mixture of the 21-monoester and 16,21-diester with the former predominating. Separation of the two components usually involves rather laborious chromatographic techniques which are clearly not desirable in large scale commercial manufacture. Since the C_{21} monoester contains the 16α, 17α-*cis* glycol grouping while the 16,21-diester does not, it seemed probable that the former should be easily solubilized by alkaline borate solutions. The proposed procedure for preparing 21-monoesters and 16,21-mixed esters via preferential borate solubility is outlined in scheme B.

Following the scheme outlined above, the 21-monoacetates, 21-monopropionates, 21-monobutyrates, and 21-monobenzoates of 9α-fluoro-16α-hydroxy hydrocortisone (V) and triamcinolone (XI) were prepared. These were, in general, characterized by their infrared spectra. Finally, when triamcinolone-21-monoacetate was treated with butyric anhydride, the mixed ester triamcinolone-21-monoacetate-16-monobutyrate was obtained.

Anal.—*Calcd.* for $C_{27}H_{33}FO_8$ (506.55): C, 64.01; H, 6.97; F, 3.75. Found: C, 64.26; H, 7.25; F, 3.94.

It should be pointed out that the separation technique described above is clearly not applicable to esters which, by nature of a functional group, would be soluble in alkali (i.e., hemiacid esters). In a future publication a general procedure will be described that may be employed to prepare 21-monoesters of all types.

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Effect of Calcium Succinate and Calcium Carbonate on the Stability of Aspirin Tablets

BY M. R. NAZARETH† and C. LEE HUYCK‡

The effect of calcium carbonate and calcium succinate on the stability of aspirin tablets containing corn starch and talc were studied over a period of eight weeks. This study shows that aspirin in the absence of moisture is stable in the presence of calcium carbonate, but unstable in the presence of calcium succinate.

THE USUAL depressant effect on tissue metabolism and respiration shown by aspirin, salicylates, etc. when administered in large and protracted doses such as in the treatment of rheumatic fever, rheumatoid arthritis, and rheumatism can be overcome by combining the above mentioned drugs with succinic acid or sodium or calcium succinate (1). Tablets containing 2.8 gr. of calcium succinate and 3.7 gr. of aspirin administered at a dosage of 8 to 15 tablets daily for two months and more showed none of the usual toxic effects of aspirin when administered alone in the same amounts (1).

This work was undertaken to determine the effect of calcium succinate on the stability of aspirin-starch mixtures. The effect of calcium carbonate on the stability of aspirin-starch mixtures was also studied.

Edwards, *et al.* (2), reported that aspirin hydro-

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lyzes readily in the presence of water and therefore its pharmaceutical preparations will contain free salicylic acid unless produced and stored in an environment free from moisture.

Ribeiro, *et al.* (3), found that certain lubricants, noticeably stearates, increased the degree of decomposition of aspirin contained in aspirin-phenacetin-caffeine tablets. Moderate water content and the pressure of the ordinary tablet machine is without effect.

Yamamoto and Takahashi (4) reported high temperature at low humidity hydrolyzes aspirin considerably. The effect of external pressure is negligible but the presence of a salt of a weak acid (calcium glycerophosphate, sodium salicylate, etc.) accelerated the decomposition of aspirin, especially in high humidity.

Hydrolysis of acetylsalicylic acid is prevented by the addition of approximately 2.5 per cent by weight of a nontoxic acid phosphate, e.g., NaH_2PO_4, as a buffer (5).

Kral, Kylarova, and Stastnikova (6) reported that 1 per cent calcium chloride stabilized calcium acetylsalicylate under normal conditions for eighteen months and for twelve to fifteen months in a humid atmosphere.

Various methods (7–25) have been used for the analysis of aspirin either as aspirin or salicylic acid in pharmaceuticals. The method of analysis used in this study was selected for its rapidity, simplicity, and fair accuracy.

EXPERIMENTAL

Aspirin tablets I were made by the slugging method. Aspirin and starch were mixed, slugged, and broken into granules by use of a No. 14 screen, followed by compression using a three-eighth inch punch. The tablet weighted 0.265 Gm.

Aspirin tablets II were made from granules of calcium carbonate prepared from powdered calcium carbonate plus 10% starch paste. The granules were dried and the moisture, as determined using a Cenco infrared moisture balance, was found to be nearly 0.1%. Aspirin and starch were mixed, slugged, broken into granules by use of a No. 14 screen, mixed with talc and calcium carbonate granules, and compressed, using a seven-sixteenth inch punch, into tablets weighing 0.355 Gm.

Aspirin tablets III were made by preparing granules of calcium succinate with 10% starch paste. The granules were dried and the moisture content was found to be approximately 0.1%. Aspirin and starch were mixed, slugged, and broken into granules by use of a No. 14 screen. The granules were mixed with talc and calcium succinate granules and compressed, using a one-half inch punch, into tablets weighing 0.479 Gm.

Aspirin tablets IV were made by mixing aspirin and starch, slugging the mixture, and breaking the slugs into granules with a No. 14 screen. The granules of calcium succinate and calcium carbonate were mixed as in II and III with the other granules and talc and compressed on a one-half inch punch into tablets weighing 0.570 Gm.

Table I.—Formulas in Grams for 1,000 Aspirin Tablets

Ingredients	I	II	III	IV
Aspirin	240.0	240.0	240.0	240.0
Calcium succinate[a]	...	...	182.0	182.0
Calcium carbonate	...	72.5	...	72.5
Starch	25.0	31.5	43.0	49.5
Talc	...	11.0	14.0	26.0
Total	265.0	355.0	479.0	570.0

[a] Furnished through the courtesy of S. B. Penick & Co., New York, N. Y.

Samples of I, II, III, and IV were kept under refrigeration (9–12°), room temperature (25–30°), and 45°. For formulas of samples, see Table I.

Method of Analysis.—The method of analysis used was essentially that of Pankratz and Bandelin (16). A standard curve was prepared by plotting the absorbance of known concentrations of pure salicylic acid at 525 mμ blanked against 20% ethyl alcohol, using a Coleman junior spectrophotometer model 6.

The free salicylic acid in aspirin used in the formulations was found to be 0.05%. The amount of aspirin/tablet in I, II, III, and IV was determined as follows: Twenty tablets were accurately weighed and the average weight per tablet recorded. The tablets were powdered and exactly 1.500 Gm. of the powdered sample was transferred to an Erlenmeyer flask with 25 ml. ethyl alcohol. The flask was well shaken, allowed to stand for ten minutes, and the solution was filtered clear. Twenty-five milliliters of 10% potassium hydroxide solution was added and the mixture was allowed to stand, lightly stoppered on a steam bath, for thirty minutes. It was then cooled and transferred to a 200-ml. volumetric flask. Five milliliters of concentrated hydrochloric acid was added with sufficient distilled water to make up to volume. Ten milliliters of this solution was diluted to exactly 500 ml. A 25-ml. aliquot of this dilution was transferred to a 50-ml. volumetric flask, 10 ml. of ethyl alcohol and 5 ml. of ferric nitrate reagent were added with sufficient distilled water to volume. The absorbance of this solution at 525 mμ was read and compared with the standard. The amount of aspirin/tablet was then determined using the standard curve as reference. The aspirin content in mg. was found to be I, 240.0; II, 240.5; III, 241.0; and IV, 241.0 mg./tablet, and was taken to be 240 mg./tablet for all further calculations.

The free salicylic acid present in the freshly prepared tablets was determined as follows: Twenty tablets were accurately weighed and powdered. Fifteen milliliters of ethyl alcohol was added to 0.500 Gm. of the powdered sample in an Erlenmeyer flask. The flask was shaken well and allowed to stand for ten minutes after which the solution was immediately filtered clear into a 100-ml. volumetric flask. Five milliliters of ferric nitrate reagent was added and distilled water added to volume. The absorbance of the solution was then read at 525 mμ when blanked against 20% ethyl alcohol. The amount of free salicylic acid present was read from the standard curve and the per cent labeled amount recorded.

TABLE II.—MG. DECOMPOSED ASPIRIN/TABLET UNDER REFRIGERATION, ROOM TEMPERATURE, AND 45° OVER AN EIGHT-WEEK AGING PERIOD

Time in Weeks	I			II			III			IV		
	Ref.[a]	R. T.[b]	45°C.	Ref.	R. T.	45°C.	Ref.	R. T.	45°C.	Ref.	R. T.	45°C.
Initial	0.30	0.30	0.30	0.70	0.70	0.70	0.90	0.90	0.90	0.90	0.90	0.90
1	0.35	0.37	0.40	0.72	0.89	1.63	0.95	1.08	4.90	0.97	1.34	28.70
2	0.37	0.38	0.45	0.73	1.37	2.87	0.95	1.40	20.60	1.01	1.73	41.70
3	0.39	0.56	0.73	0.88	1.67	10.02	1.00	1.86	195.00	1.04	1.84	119.00
4	0.50	0.60	0.92	0.94	1.99	16.40	1.18	2.07	195.00	1.47	2.15	171.00
5	0.54	0.66	0.99	1.03	2.19	23.80	1.30	2.12	204.00	1.55	2.38	226.00
6	0.57	0.77	1.09	1.07	2.41	28.40	1.37	2.19	204.00	1.56	2.54	233.00
7	0.59	0.80	1.17	1.11	2.50	31.00	1.50	2.26	204.00	1.56	2.61	233.00
8	0.74	0.83	1.22	1.22	2.65	33.50	1.63	2.37	204.00	1.63	2.67	233.00

[a] Ref., refrigerated 9 to 12°. [b] R. T., room temperature 25 to 30°.

TABLE III.—PER CENT ASPIRIN/TABLET UNDER REFRIGERATION, ROOM TEMPERATURE, AND 45° OVER AN EIGHT-WEEK AGING PERIOD

Time in Weeks	I			II			III			IV		
	Ref.[a]	R. T.[b]	45°C.	Ref.	R. T.	45°C.	Ref.	R. T.	45°C.	Ref.	R. T.	45°C.
Initial	99.9	99.9	99.9	99.8	99.8	99.8	99.7	99.7	99.7	99.7	99.7	99.7
1	99.9	99.9	99.9	99.8	99.7	99.5	99.6	99.5	98.0	99.6	99.5	88.1
2	99.9	99.9	99.9	99.7	99.5	98.9	99.6	99.3	91.5	99.6	99.4	82.7
3	99.9	99.8	99.7	99.7	99.5	96.0	99.6	99.5	19.0	99.6	99.4	50.0
4	99.9	99.8	99.6	99.7	99.3	94.0	99.6	99.2	19.0	99.5	99.0	29.2
5	99.8	99.7	99.6	99.6	99.0	90.2	99.5	99.1	11.5	99.4	99.0	5.7
6	99.7	99.7	99.6	99.6	99.0	88.2	99.5	99.1	11.5	99.4	99.0	3.3
7	99.7	99.6	99.5	99.5	98.9	87.3	99.5	99.0	11.5	99.4	98.8	3.3
8	99.7	99.6	99.5	99.5	98.8	86.0	99.4	99.0	11.5	99.4	98.8	3.3

[a] Ref., refrigerated 9–12°. [b] R. T., room temperature 25–30°.

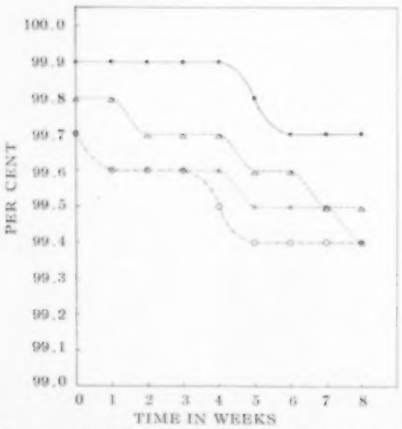

Fig. 1.—Stability of aspirin in tablets at 9–12°. ●——●, formula I, control; △- - -△, formula II, with 20.4% calcium carbonate; ×. . . ×, formula III, with 38.0% calcium succinate; ○- - -○, formula IV, with 12.7% calcium carbonate and 32.0% calcium succinate.

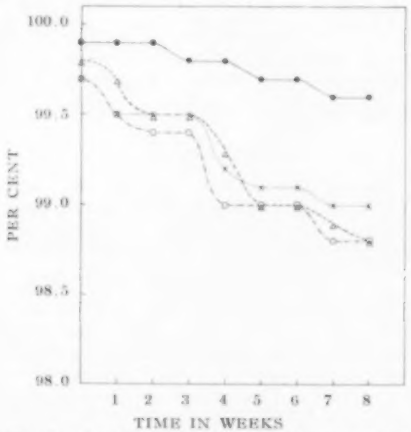

Fig. 2.—Stability of aspirin in tablets at 25–30°. ●——●, formula I; △- - -△, formula II; ×. . . .×, formula III; ○— - —○ formula IV.

The per cent labeled amount of I, II, III, and IV at the three temperature levels was determined weekly throughout the period of study and these results are recorded in Tables II and III. In the case of tablets III and IV at 45° there was difficulty in separating whole tablets after three weeks and four weeks, respectively, because the tablets fused together at this temperature. Estimation was therefore based on the average weights of earlier determinations.

DISCUSSION

Under refrigeration I, II, III, and IV showed very little deterioration and the final values after an eight week period were I, 99.7%; II, 99.5%; III, 99.4%; and IV, 99.4% of the labeled amount.

At room temperature, deterioration was not appreciable and the final values were I, 99.6%; II, 98.8%; III, 99.0%; and IV, 98.8% of the labeled amount.

At 45°, I kept rather well, the final value being 99.5%; II decreased slowly to 86.0%; while III and IV showed marked deterioration with final

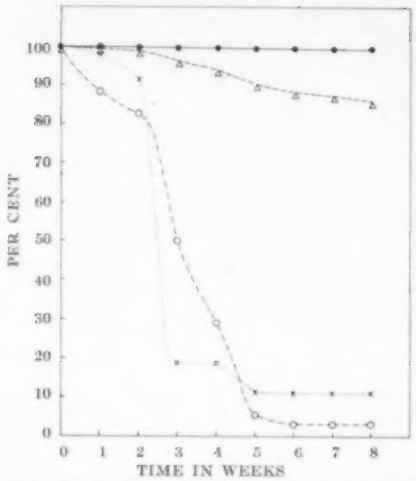

Fig. 3.—Stability of aspirin at 45°. ●——●, formula I; △- - -△, formula II; ×. . . .×, formula III; ○— - —○, formula IV.

values of 11.5 and 3.3% of the labeled amount, respectively. Tablets II and IV took on a slightly pinkish tint after two weeks, which is probably due to an iron salicylate complex. A trace of iron was detected as an impurity in the calcium carbonate used in the formulations. In the case of II, III, and IV needle shaped crystals of salicylic acid were noticed on the sides and neck of the containers when the level fell below 94.0% and a strong acetic acid odor prevailed. Tablets III and IV fused together after three and four weeks, respectively. The deterioration in III and IV at 45° appeared to be autocatalytic judging by the rather rapidly increasing rate of deterioration. When the values of III and IV reach 11.5 and 3.3%, respectively, there is an apparent equilibrium and no further deterioration takes place. For the stability of aspirin at the different temperatures, see Figs. 1, 2, and 3.

This study shows that aspirin is unstable in the presence of calcium succinate. This is in agreement with Yamamoto and Takahashi (4) who found that the presence of a salt of a weak acid accelerates the decomposition of aspirin. Calcium carbonate does not appear to cause any marked deterioration of aspirin.

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Procedures for Assay and Stability Determination of Phenazocine Hydrobromide in Pharmaceutical Preparations

By IRVING B. EISDORFER, JOAN G. ROSEN, and WILLIAM C. ELLENBOGEN

Although quite stable in acid solution, phenazocine hydrobromide is appreciably decomposed by heat in alkaline solution. The products of this decomposition reaction have been isolated and identified as styrene and the "des-phenethyl" analog of phenazocine hydrobromide. Ultraviolet absorption and nonaqueous titration procedures are presented for determining the undecomposed compound after separation from its decomposition products. These procedures are suitable for assaying and determining the stability of phenazocine hydrobromide in various pharmaceutical preparations.

PHENAZOCINE HYDROBROMIDE[1] is a new potent analgetic recently discovered at the National Institutes of Health by May and Eddy (1). Due to its high activity and relatively low addictive liability, it shows promise of becoming an important agent in the treatment of certain severe pain states (2).

In connection with the development of stable dosage forms for dispensing phenazocine hydrobromide, it was necessary to develop analytical procedures for assaying and determining the stability of this compound after storage under accelerated aging conditions. In order to develop suitable stability methods, it was necessary to determine the course of the primary decomposition reaction. This report describes the isolation and identification of the decomposition products and some analytical procedures for determining the compounds in decomposed samples.

EXPERIMENTAL

Isolation and Identification of Decomposition Products.—Preliminary attempts to decompose phenazocine hydrobromide were distinctly unpromising. Even after forty-eight hours at 100°, the chemical showed no change in U. V. or I. R. absorption, melting point, or paper chromatography. Similarly, acidified aqueous solutions of the salt showed no evidence of decomposition even after being autoclaved at 120° for six hours. The free base chemical, however, showed distinct changes in U. V., I. R., melting point, and paper chromatography after being heated for forty-eight hours at 100°. Neutral or alkaline solutions of the salt also showed similar evidences of decomposition after being autoclaved for 120° for six hours.

The infrared absorption curve of the partially decomposed compound showed a minor absorption peak at 3.05 μ which appeared to indicate formation of a secondary amine. The U. V. absorption curve of the decomposed solution showed the presence of a new compound with a maximum absorption in the region of 249 mμ, otherwise the curve was unchanged (Fig. 1). The paper chromatogram of the decomposed sample showed the presence of a new compound which gave a positive spray reaction for secondary or tertiary amine (potassium iodoplatinate reagent) but not for a primary amine (ninhydrin reagent).

In order for phenazocine to form a secondary amine on decomposition, it would have to suffer ring opening or loss of the phenethyl group at the nitrogen. If the phenethyl group were lost, it appeared likely that it would form the hydrocarbon styrene and leave behind the "des-phenethyl" secondary amine. If, instead, the ring opened, the resulting molecule might contain a new double bond, perhaps in the terminal position, but retain the phenethyl grouping (Fig. 2).

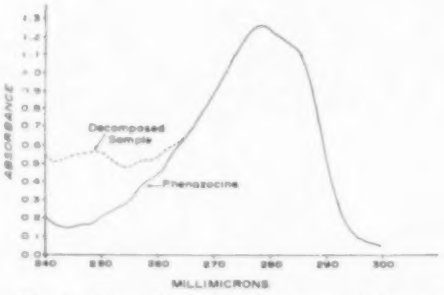

Fig. 1.—Ultraviolet absorption curve of phenazocine and of a heat-decomposed sample run in acidified alcohol.

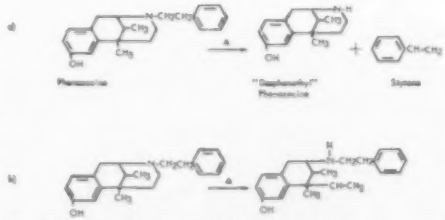

Fig. 2.—Possible alternate decomposition pathways.

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[1] The material used in this study was Prinadol, Smith Kline and French brand of phenazocine hydrobromide.

To determine which of these hypotheses was correct, an attempt was made to isolate styrene from a decomposed phenazocine sample by extraction with cyclohexane after acidification. The extract did, in fact, show the presence of a substance with a U. V. absorption curve strongly resembling that of styrene (maximum at 249 mμ) (Fig. 3). The presence of styrene in the extract was further confirmed by vapor phase chromatography which showed the presence of a band corresponding with that of styrene. It was not found possible to extract sufficient styrene from the decomposed solutions for a satisfactory I. R. identification, probably because most of the styrene polymerized on heating and became insoluble in the extraction solvent.

In order to confirm the identity of the residual secondary amine, it was necessary to separate it from the undecomposed phenazocine. Fortunately, an authentic sample of the postulated secondary amine (the "des-phenethyl" amine) was available since it is an intermediate in the synthesis of phenazocine hydrobromide. Two procedures were worked out for separating the secondary amine from phenazocine. One was based on the extraction of phenazocine from an alkaline solution of the mixture with cyclohexane, leaving the secondary amine behind in the aqueous phase. The other was based on the extraction of phenazocine hydrobromide from a 0.1 *N* hydrochloric acid solution with chloroform, again leaving the secondary amine behind in the aqueous phase. Using these separation procedures, it was relatively easy to isolate sufficient of the secondary amine from a decomposed sample to establish by paper chromatography that it was identical with the authentic "des-phenethyl" secondary amine (Fig. 4). The U. V. and I. R. absorption studies further confirmed this fact.

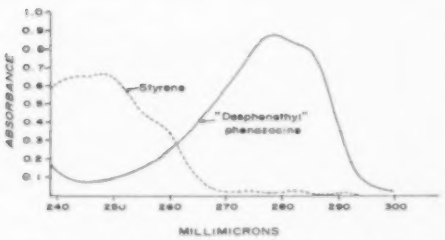

Fig. 3.—Ultraviolet absorption curves of "desphenethyl" phenazocine and styrene run in acidified alcohol.

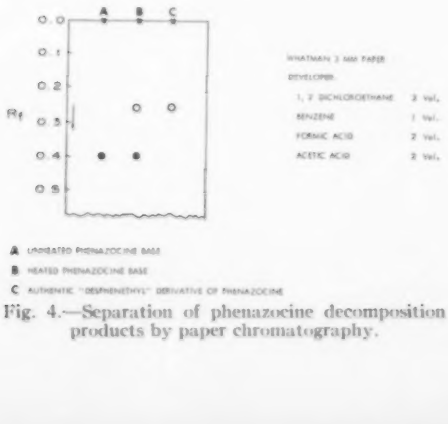

Fig. 4.—Separation of phenazocine decomposition products by paper chromatography.

Paper chromatograms of decomposed samples often show the presence of small amounts of decomposition products other than those described above. This would indicate that the decomposition reaction described above is not the only decomposition pathway, although undoubtedly the predominant one.

In order to determine the stability of phenazocine hydrobromide in various pharmaceutical formulations, specific analytical procedures were required for determining the intact compound in the presence of, or after separation from its decomposition products. These procedures are described in detail in the following sections.

STABILITY ASSAY METHODS

A. Ultraviolet Absorption Procedure.—Transfer an accurately measured sample containing about 20 mg. of phenazocine hydrobromide to a 250-ml. separatory funnel. Dissolve in, or dilute with, 0.1 *N* hydrochloric acid to a volume of about 25 ml. Add 5 ml. of 5% (w/v) sodium hydroxide and 2 Gm. of sodium sulfate, and mix until the salt is dissolved. Extract with four 25-ml. portions of cyclohexane, then re-extract the combined cyclohexane solution with four 20-ml. portions of 0.1 *N* hydrochloric acid. Collect the combined extract in a 100-ml. volumetric flask and dilute to volume with 0.1 *N* hydrochloric acid. Transfer a 25-ml. aliquot to each of two 50-ml. volumetric flasks. Dilute one to volume with 0.1 *N* hydrochloric acid in 95% alcohol, and dilute the other to volume (after cooling to room temperature) with 0.2 *N* potassium hydroxide in 95% alcohol. Determine the absorbance (*A*) of each solution in a 1-cm. cell at 301 and 330 mμ, using the appropriate solvent as a blank in each case. In a similar manner, transfer a 25-ml. aliquot of a standard solution containing 0.2 mg./ml. of phenazocine hydrobromide in 0.1 *N* hydrochloric acid to each of two 50-ml. volumetric flasks. Dilute one to volume with 0.1 *N* hydrochloric acid in 95% alcohol and dilute the other to volume with 0.2 *N* potassium hydroxide in 95% alcohol, then proceed as before.

TABLE I.—SPECIFIC ABSORPTIVITY[a] OF PHENAZOCINE HYDROBROMIDE

Wavelength, mμ	Specific Absorptivity, *A*/mg./ml. Acid Solution	Basic Solution
301	0.0289	7.434
330	Nil	Nil

[a] Determined with a Cary recording spectrophotometer.

TABLE II.—RECOVERY OF PHENAZOCINE FROM KNOWN MIXTURES

Added		Found, mg., Phenazocine HBr	
Phenazocine Hydrobromide, mg.	"Des-phenethyl" Derivative, mg.	Procedure A	Procedure B
20	..	19.9, 19.8	20.0, 20.1
20	..	19.8, 19.8	20.2, 20.2
20	5	20.1, 20.3	20.2, 20.3
20	10	20.2, 20.2	20.3, 20.2

Table III.—Recovery of Phenazocine from Pharmaceutical Formulations

Sample	Age	Claim	Found	
			Procedure A	Procedure B
Ampul	Original	2 mg./ml.	1.99, 1.98	2.00, 2.02
	6 mo., 37°	2 mg./ml.	1.96, 1.98	1.97, 1.98
M.D.V.	Original	2 mg./ml.	...	1.99, 1.98
	6 mo., 37°	2 mg./ml.	...	1.96, 1.97
Ampul (unbuffered)	Artificially decomposed	0.1 mg./ml.	0.092, 0.091	
Solid formulation	Original	2.5 mg./capsule	2.48, 2.50	...
	6 mo., R.T.	2.5 mg./capsule	2.48, 2.48	

phenazocine hydrobromide, mg./ sample wt. or volume =

$$\frac{[(A_{301} - A_{330})_{base} - (A_{301} - A_{330})_{acid}]\text{sample}}{[(A_{301} - A_{330})_{base} - (A_{301} - A_{330})_{acid}]\text{std.}} \times \frac{\text{concn. of std. (mg./ml.)} \times 100}{\text{sample wt. or volume}}$$

B. Titration Procedure.—Transfer an accurately measured sample containing about 100 mg. of phenazocine hydrobromide to a separatory funnel containing 50 ml. of 0.1 *N* hydrochloric acid. Extract with four 30-ml. portions of chloroform. Filter the combined chloroform extract through a pledget of cotton wet with chloroform into a clean dry titration flask. Rinse the pledget and funnel thoroughly with excess chloroform, catching the rinsings in the flask. Evaporate off the chloroform on the steam bath with the aid of a stream of dry air. Take the residue up in 25 ml. of glacial acetic acid, add 10 ml. of 6% mercuric acetate in acetic acid, and a few drops of 1% crystal violet in acetic acid. Titrate with 0.02 *N* perchloric acid in glacial acetic acid to the first disappearance of the red when viewed through a yellow filter. Determine the blank correction by titrating an equivalent amount of glacial acetic acid in the same manner.

phenazocine hydrobromide, mg. =
net titration (ml.) × *N* $HClO_4$ × 402.3

RESULTS AND DISCUSSION

While the procedures are equally applicable to most ampul, multidosage vial, and solid formulations (after suitable extraction), one or the other of the procedures might be preferable under certain circumstances. Procedure A, for instance, would be preferable when time or sample conservation were important considerations. It would also be desirable to use procedure A where the selectivity of the U. V. absorption curve for identity confirmation was important. Due to the use of the "differential absorbance" calculation, procedure A suffers little interference from most weak or even moderately strong U. V. absorbers, even though they may find their way through the solvent extraction barrier. Phenols and other strong absorbers are likely to interfere with this procedure if they come through the extraction procedure.

Procedure B is the procedure of choice for samples containing U. V. absorbers that interfere with procedure A. Except for the relatively few organic bases whose halide salts are extractable into chloroform from acid solution, few interferences have been encountered for procedure B. Certainly where instrumentation or reference compounds are not available, procedure B must be employed. Although it is strongly recommended that pure phenazocine hydrobromide be used as a reference standard in procedure A and that the appropriate specific absorptivity be determined by each operator with his own instrument, these values are listed in Table I for general reference purposes.

When mixtures of phenazocine hydrobromide and the "des-phenethyl" amine were analyzed by both procedures, the results shown in Table II were obtained.

Ampul solutions containing phenazocine hydrobromide, sodium citrate, sodium phosphate, citric acid, sodium chloride, and propylene glycol were assayed as originally prepared and after various storage intervals by both procedures. The results obtained are shown in Table III. Original and aged samples of multiple-dose vial formulations, which contain benzyl alcohol in addition to the above ingredients, were assayed by procedure B. These results and those resulting from the assay of an original and aged solid formulation are also shown in Table III.

These data indicate that typical formulations containing phenazocine hydrobromide can be assayed with an accuracy of approximately ±1% by either method. The procedures appear to be entirely adequate for assaying decomposed or stored samples.

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Dietary Feeding of Propylene Glycol Ethers of Methylcellulose to Rats

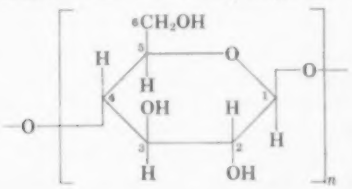
By D. D. McCOLLISTER†, F. OYEN†, and G. K. GREMINGER, Jr.

Patents, published literature, and commercial usage have established the utility of the propylene glycol ether of methylcellulose (hydroxypropyl methylcellulose) as a food additive and as a formulation aid in drug products. Aqueous solutions of this cellulose gum, like methylcellulose, will gel upon heating. Feeding studies on Methocel, Methocel 60HG, and Methocel 65HG have previously been published. The results of feeding Methocel 70HG and Methocel 90HG in the diet of rats at levels of 0.3, 1.0, 3.0, 10.0, and 20.0 per cent for periods of ninety and eighty-four days, respectively, confirm previous chronic oral toxicity studies that the propylene glycol ether of methylcellulose is extremely low in repeated oral toxicity and that it can be used safely in the drug, food, and pharmaceutical fields where Methocel and Methocel 65HG have been found suitable.

GUMS HAVE BEEN used in food and drug preparations since earliest times. The first products were of natural origin and were used in either the "as found" or isolated states. Advancing technology has required more rigid control of gum properties and, in addition, has created a demand for products of increased utility. Methylcellulose[1] and its modification, propylene glycol ether of methylcellulose (hydroxypropyl methylcellulose)[1] have provided combinations of gum properties which have greatly expanded the industrial use of synthetic gums.

The propylene glycol ether of methylcellulose is a water-soluble ether of cellulose containing both methyl and propylene glycol ether groups attached to the anhydroglucose ring of cellulose. The propylene glycol ether of methylcellulose may also be described as hydroxypropyl methylcellulose.

The polymeric backbone of the propylene glycol ether of methylcellulose is provided by cellulose. The repeating structural unit of the cellulose chain is the anhydroglucose unit:



This anhydroglucose unit contains one primary hydroxyl group in the 6-position and two secondary hydroxyl groups in the 2- and 3-positions. Thus, the propylene glycol ether of methylcellulose is fundamentally a carbohydrate derivative containing a basic structure of condensed glucose rings.

As mentioned above, three positions are available for substitution on the anhydroglucose ring of cellulose. Spurlin (1) suggested that the distribution of substituents among these three positions is random and may be calculated by statistical methods. Thus, all of the hydroxyls may have an equal chance to react and the substituents will be distributed among the available positions according to the laws of probability.

The methoxyl portion of the substitution of the materials used in this research is the major part, comprising 84.0 to 93.3 per cent of the substituent groups attached to the anhydroglucose ring. The propylene glycol ether degree of substitution (D. S.) is a minor portion of the total substitution and it is present in a range of only 0.07 to 0.3 D. S. The total D. S. (methoxyl D. S. + propylene glycol ether D. S.) does not exceed 2.2. While the amount of the propylene glycol ether substitution is small, it does have an important effect upon the functional and beneficial properties of the basic methylcellulose.

The pharmacology of methylcellulose is well established (2–8). The fundamental facts of the structure and the properties of the methyl ether moiety of propylene glycol ether of methylcellulose are identical to those of methylcellulose. Methylcellulose has been used as a direct additive to foods for many years and is generally recognized as being safe (9).

In addition, the definitions and standards of identity for French and salad dressings have been amended to permit the use of methylcellulose U. S. P. as an optional emulsifying ingredient (10).

Propylene glycol ether of methylcellulose is made in several types to achieve specific ranges of thermal gel points for various applications.

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† Biochemical Research Laboratory.

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[1] The Dow Chemical Co. supplies methylcellulose as Methocel MC and propylene glycol ethers of methylcellulose as Methocel HG.

TABLE I.—CHARACTERISTICS OF DIFFERENT PROPYLENE GLYCOL ETHERS OF METHYLCELLULOSE

Methocel HG Type	Methoxyl[a] Range of D. S.	Methoxyl[a] Av. D. S.	Propylene Glycol Ether Range of D. S.	Propylene Glycol Ether Av. D. S.	Thermal Gel Point for 2% Soln. of 4,000 cps., ° C.
Methocel MC U. S. P.	1.64–1.92	1.78	.....	...	~50
Methocel 60HG	1.67–1.82	1.75	0.17–0.30	0.24	55–60
Methocel 65HG	1.61–1.75	1.68	0.10–0.18	0.14	60–65
Methocel 70HG	1.42–1.62	1.52	0.07–0.13	0.10	66–72
Methocel 90HG	1.08–1.42	1.25	0.10–0.30	0.20	Over 85

[a] Determined by A.S.T.M. method D1347-56.

TABLE II.—FOOD CONSUMPTION AND FOOD EFFICIENCY RECORDS DURING THE FIRST THIRTY-FIVE DAYS FOR GROUPS OF MALE AND FEMALE RATS MAINTAINED FOR NINETY DAYS ON DIETS CONTAINING METHOCEL 70 HG

Sex	% In Diet	Average Body Weight, (Gm.) Initial	Average Body Weight, (Gm.) At the End of 35-Day Period	Average Body Weight Gained, Gm.	Average Food Consumption per Rat, During the 35-Day Period Gm./Day	Average Food Consumption per Rat, During the 35-Day Period Total, Gm.	Food Efficiency[a]
M	Control	121	254	133	17.4	609.0	4.58
M	20	122	185	63	16.6	581.0	9.22
M	10	121	235	114	19.2	672.0	5.89
M	3	122	250	128	18.2	637.0	4.98
M	1	122	242	120	16.8	588.0	4.90
M	0.3	123	261	138	17.5	612.5	4.44
F	Control	101	164	63	13.5	472.5	7.50
F	20	102	145	43	13.7	479.5	11.51
F	10	101	166	65	13.7	479.5	7.38
F	3	101	167	66	14.4	504.0	7.64
F	1	101	171	70	14.1	493.5	7.05
F	0.3	102	168	66	13.7	479.5	7.27

[a] Grams of food eaten per gram of body weight gained.

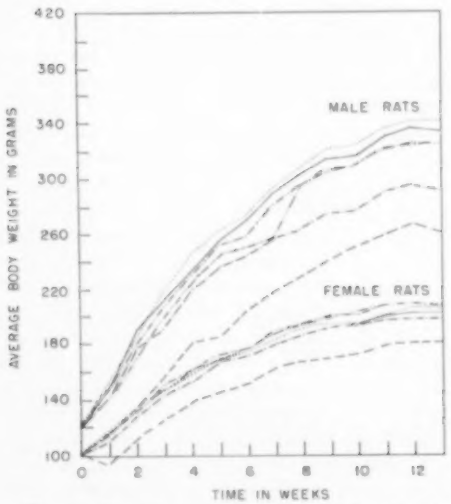

Fig. 1.—Growth curves of male and female rats maintained for ninety days on Methocel 70HG. ——, Control; - - -, 20%; –·–·–, 10%; –··–··–, 3%, –···–···–, 1%; ...., 0.3%.

The letters "HG" refer to "high gel" and the number before HG refers to the approximate temperature in ° C. of the thermal gel point, as given in Table I. For example, there has been considerable demand for a product with the functional properties of methylcellulose which would have a thermal gel point high enough to avoid gelation during pasteurization. The thermal gel point can be increased by decreasing the methoxyl content. As this is done, water solubility decreases faster than the gel point increases; therefore, some other type of substitution must be added to maintain water solubility. This is the function of the small amount of hydrophilic propylene glycol ether substitution. Therefore, although these changes in D. S. are small, encompassing a range of only 0.07 D. S. to 0.3 D. S., they can have a critical effect upon the functional properties desired in a specific propylene glycol ether of methylcellulose.

Extensive toxicological studies have also been conducted on the propylene glycol ether of methylcellulose. Data on Methocel 60HG and Methocel 65HG have previously been published (11–13). This paper presents additional data on the toxicology of Methocel 70HG and 90HG which completes the series presented in Table I.

EXPERIMENTAL

Description of Materials.—Methocel 70HG is a mixed methyl and hydroxypropyl ether of cellulose similar to Methocel 60HG and Methocel 65HG. It has a higher gel point than either of the other two materials. This higher gel point and its water

TABLE III.—FINAL AVERAGE BODY AND ORGAN WEIGHTS AND HEMATOCRIT DETERMINATIONS FOR MALE AND FEMALE RATS MAINTAINED NINETY DAYS ON DIETS CONTAINING METHOCEL 70HG

No. Rats	Sex	% In Diet	Average Body Weight, Gm.[a]	Average Organ Weight, Gm./100 Gm. Body Weight						Packed Cell Volume, Hematocrit
				Lung	Heart	Liver	Kidney	Spleen	Testes	
10	M	Control	317	0.59	0.37	2.72	0.74	0.32	0.95	49.5
7	M	20	237[b]	0.62	0.38	2.70	0.73	0.31	1.24	46.0
8	M	10	273[c]	0.60	0.37	2.81	0.79	0.30	1.08	44.5
10	M	3	308	0.63	0.36	2.79	0.74	0.28	1.03	48.5
10	M	1	307	0.57	0.35	2.72	0.72	0.31	0.98	44.5
10	M	0.3	324	0.55	0.36	2.66	0.72	0.30	0.93	41.0
10	F	Control	189	0.69	0.41	2.84	0.75	0.32	...	46.0
7	F	20	158[c]	0.78	0.42	3.03	0.80	0.37	...	50.5
9	F	10	181	0.77	0.41	2.98	0.81	0.40	...	44.0
10	F	3	192	0.79	0.41	3.05	0.79	0.34	...	44.0
10	F	1	193	0.75	0.42	2.85	0.80	0.36	...	44.0
9	F	0.3	192	0.73	0.41	2.93	0.77	0.32	...	47.5

[a] Animals fasted overnight. [b] Moderate growth depression. [c] Slight growth depression.

TABLE IV.—FINAL AVERAGE BODY AND ORGAN WEIGHTS FOR MALE AND FEMALE RATS MAINTAINED FOR NINETY DAYS ON DIETS CONTAINING METHOCEL 70HG

No. Rats	Sex	% In Diet	Average Body Weight, Gm.[a]	Average Organ Weights, Gm.					
				Lung	Heart	Liver	Kidney	Spleen	Testes
10	M	Control	317	1.86	1.16	8.64	2.35	1.01	3.01
7	M	20	237[b]	1.46	0.91	6.41	1.74	0.74	2.93
8	M	10	273[c]	1.62	1.02	7.68	2.15	0.84	2.96
10	M	3	308	1.95	1.10	8.59	2.26	0.86	3.18
10	M	1	307	1.76	1.06	8.35	2.20	0.94	3.01
10	M	0.3	324	1.78	1.18	8.61	2.34	0.98	3.02
10	F	Control	189	1.31	0.77	5.36	1.42	0.61	...
7	F	20	158[c]	1.24	0.66	4.78	1.26	0.59	...
9	F	10	181	1.40	0.74	5.40	1.46	0.72	...
10	F	3	192	1.51	0.78	5.86	1.53	0.65	...
10	F	1	193	1.44	0.81	5.50	1.54	0.69	...
9	F	0.3	192	1.40	0.79	5.63	1.47	0.61	...

[a] Animals fasted overnight. [b] Moderate growth depression. [c] Slight growth depression.

solubility make it useful for certain applications in the food field. It has a high molecular weight and contains 24 to 27% methoxyl groups and 3.0 to 5.5% hydroxypropoxyl groups. In appearance, it is a white powder with a melting point of 296–307°. It is soluble in water up to 10 to 25%, is insoluble in many organic solvents, and is stable to alkalies and weak acids. It has a specific gravity of 28 to 32 pounds per cubic foot (0.45–0.51 Gm./ml.).

Methocel 90HG also is a mixed methyl and hydroxypropyl ether of cellulose similar to Methocel 60HG and Methocel 65HG with a higher gel point than either of the other two materials. It contains 19 to 24% methoxyl groups and 4 to 12% hydroxypropoxyl groups. In appearance, it is a white powder which has a melting point of 290–307°. It is soluble in water up to 10 to 25%, is insoluble in many organic solvents, and is stable to alkalies and to weak acids. Its specific gravity is 28 to 35 pounds per cubic foot (0.45 to 0.55 Gm./ml.).

Procedures.—The experimental animals used in this study were white albino rats taken from the stock colony of this laboratory. These animals were progeny of rats obtained in 1938 from the Wistar Institute and maintained as a closed colony since. Young male and female rats were selected at weaning and received the stock ration (Purina Laboratory Chow) until they were approximately forty-five days of age (70HG) or sixty days of age (90HG). At this time they were divided into well-matched groups of 10 animals of each sex and started on diets containing 0.0 (control), 1.3, 1.0, 3.0, 10.0, and 20.0% of Methocel 70HG or 90HG. The experimental diets were prepared by mixing the test materials thoroughly with ground Purina Laboratory Chow. Two rats were placed together in wire-bottom cages and they had free access to food and water at all times.

During the course of the experiment, the rats were observed frequently for changes in gross appearance and behavior. Records were kept of body weights (biweekly) and average food consumption per rat. At the end of the approximately three-month periods, all of the rats were fasted overnight, weighed, and sacrificed by decapitation.

At autopsy, gross pathological examinations were made and the lungs, heart, liver, kidneys, testes, and spleen were removed and weighed. Portions of these tissues as well as adrenals and pancreas were preserved in formalin solution. Hematoxylin-eosin stained sections of these organs were prepared for microscopic examination. At autopsy also, blood hematocrit values were obtained.

Experimental Results.—*Methocel 70HG.*—The groups of animals of both sexes receiving 0.3, 1.0, and 3.0% of Methocel 70HG in their diets for ninety days showed no evidence of adverse effects as judged by growth (see Fig. 1) and appearance, behavior, food consumption, and food efficiency, measured for a thirty-five-day period, (Tables II

Table V.—Average Grams of Food Eaten per Day by Male and Female Rats Maintained on Diets Containing Various Levels of Methocel 70HG for Ninety Days

% in Diet	Av. Food Eaten, Gm. per Male Rat per Day		Av. Food Eaten, Gm. per Female Rat per Day	
	First 6 Days	35 Days	First 6 Days	35 Days
Control	13.3	17.4	11.3	13.5
20	8.3	16.6	5.9	13.7
10	13.7	19.2	10.1	13.7
3	15.3	18.2	10.9	14.4
1	11.5	16.8	13.2	14.1
0.3	14.4	17.5	11.6	13.7

and V), mortality, average final body and organ weights (Tables III and IV), gross and microscopic examination of the tissues, and blood hematocrit values.

The female rats receiving 10% of Methocel 70HG showed no adverse effects whatsoever. However, the male rats at the same level showed slight but significant growth retardation (see Fig. 1). Three rats, two males and one female, died during the course of the experiment. Their deaths were determined to have been caused by spontaneous lung or ear infection. It was noted also that the male rats had distended abdomens and that their feces were bulky and light colored.

Three male and three female rats receiving 20% of Methocel 70HG in their diets died during the course of the experiment from indeterminant causes. Definite growth retardation was observed in both the male and female rats at this level. This was accompanied by a definite decrease in the food efficiency of the diets at this level (Table II), by poor acceptance of the diet during the first week (Table V), by an "apparent" increase in the weight of the testes of the male rat on a per cent of body weight basis (Table III). However, on an absolute weight basis there was no effect (Table IV).

The above conclusions were reached by inspection of the data. The usual statistical analyses of mean values for significance between control and treated groups were not believed needed in this case, or for the Methocel 90HG data described below.

No histopathologic effects due to the Methocel 70HG were found in the male and female rats receiving either the 10 or 20% levels.

Methocel 90HG.—Throughout the experimental period, all of the rats of both sexes appeared to be in excellent health as judged by gross appearance and behavior. Mortality records showed that all of the female rats survived the full experimental period of eighty-four days. However, some of the male rats on the experimental diets as well as in the control group died from either indeterminant causes or from upper respiratory infection. Thus, the deaths of the male rats were judged to be incidental and not due to the ingestion of the test material.

Average body weight curves (see Fig. 2) revealed a definite retardation of growth of the male rats that received the diet containing 20% of Methocel 90HG, and a slight retardation of growth at the 10% level. The growth of all the other groups of male rats and all of the female rats appeared unaffected by the ingestion of the test material.

Food consumption records (Table VI) indicate

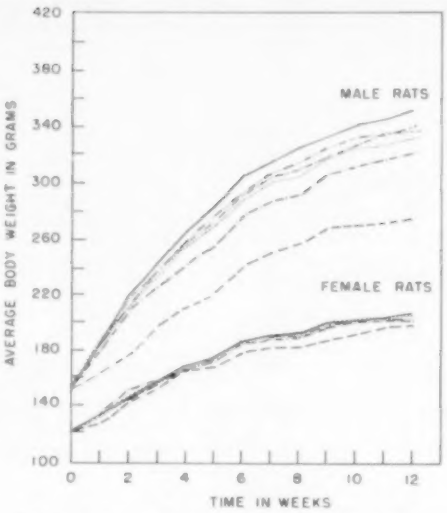

Fig. 2.—Growth curves of male and female rats maintained for eighty-four days on Methocel 90HG. ——, Control; - - -, 20%; —·—, 10%; —·—·—, 3%; —·—·—·—, 1%; · · · ·, 0.3%.

that the diet containing Methocel 90HG was well accepted. However, there is a definite decrease in food efficiency (Gm. of food eaten per Gm. of body weight gained) at the 20% level for both the males and females. This effect was most marked in the males.

The average organ weights expressed in Gm./100 Gm. of body weight (Table VII) would seem to indicate that liver, kidneys, and testes were increased for the male rats receiving the diet containing 20% Methocel 90HG. However, from the food efficiency data (Table VI) and the lack of any specific evidence of ill-effect on the tissues, it is logical to conclude that this organ weight response is consistent with that to be expected in undernourished animals due to the non-nutritive bulk of Methocel 90HG in the diet. This conclusion is substantiated by the expression of organ weights on the absolute basis (Table VIII). The female rats at the 20% levels and the animals at the 10, 3, 1, and 0.3% levels showed no significant differences in the average organ weights (Table VII).

None of the animals on the various levels (including those of the 20% levels) of Methocel 90HG showed any evidence of adverse effects as judged by gross and microscopic examination and blood hematocrit values.

DISCUSSION

The results show that the only significant effect attributable to the ingestion of Methocel 70HG or Methocel 90HG in the diet of rats was that of growth retardation at the 20% level and only in the male rats at the 10% level. The observed decrease in food efficiency, and the distention of the abdomens with the passage of bulky feces by these rats would indicate that the growth effects may well be due to the non-nutritive bulk of the

TABLE VI.—FOOD CONSUMPTION AND FOOD EFFICIENCY RECORD DURING THE FIRST THIRTY-FIVE DAYS FOR GROUPS OF MALE AND FEMALE RATS MAINTAINED FOR EIGHTY-FOUR DAYS ON DIETS CONTAINING METHOCEL 90HG

Sex	% In Diet	Average Body Weight, Gm. At End of 35-Day Period: Initial	Average Body Weight Gained, Gm.	Average Food Consumption Per Rat During 35-Day Period: Gm./Day	Total, Gm.	Food Efficiency[a]
M	Control	151	284	133	18.6	651	4.90
M	20.0	151	220	69	19.3	675.5	9.79
M	10.0	153	256	103	20.2	707	6.86
M	3.0	153	274	121	19.9	696.5	5.76
M	1.0	150	277	127	19.8	693	5.46
M	0.3	154	270	116	20.0	700	6.03
F	Control	119	174	55	14.7	514.5	9.35
F	20.0	121	168	47	16.7	584.5	12.44
F	10.0	121	172	51	15.6	546	10.71
F	3.0	119	171	52	13.4	469	9.01
F	1.0	118	174	56	13.7	479.5	8.56
F	0.3	119	172	53	14.2	497	9.38

[a] Grams of food eaten per gram of body weight gained.

TABLE VII.—FINAL AVERAGE BODY AND ORGAN WEIGHTS AND PACKED CELL VOLUME DETERMINATIONS FOR MALE AND FEMALE RATS MAINTAINED FOR 84 DAYS ON DIETS CONTAINING METHOCEL 90HG

No. Rats	Sex	% In Diet	Average Body Weight, Gm.[a]	Average Organ Weight, Gm./100 Gm. Body Weight: Lung	Heart	Liver	Kidney	Spleen	Testes	Packed Cell Volume
8	M	Control	337	0.64	0.33	2.75	0.73	0.32	0.92	45.0
8	M	20	241[b]	0.60	0.35	2.88	0.77	0.32	1.19	47.5
10	M	10	303[c]	0.54	0.35	2.84	0.77	0.32	1.02	45.0
9	M	3	320	0.64	0.32	2.75	0.75	0.34	0.99	...
9	M	1	323	0.60	0.35	2.66	0.75	0.32	0.99	...
10	M	0.3	316	0.60	0.36	2.76	0.74	0.30	1.09	...
10	F	Control	194	0.73	0.41	3.04	0.82	0.41	...	44.5
10	F	20	179	0.71	0.42	3.12	0.87	0.37	...	43.0
10	F	10	188	0.69	0.42	2.98	0.81	0.30	...	45.0
10	F	3	189	0.73	0.40	2.83	0.79	0.35	...	46.0
10	F	1	191	0.75	0.40	2.94	0.77	0.38	...	...
10	F	0.3	193	0.67	0.40	2.89	0.77	0.38	...	...

[a] Animals fasted overnight. [b] Moderate growth depression. [c] Slight growth depression.

TABLE VIII.—FINAL AVERAGE BODY AND ORGAN WEIGHTS FOR MALE AND FEMALE RATS MAINTAINED FOR EIGHTY-FOUR DAYS ON DIETS CONTAINING METHOCEL 90HG

No. of Rats	Sex	% in Diet	Average Body Weight, Gm.[a]	Average Organ Weights Absolute, Gm.: Liver	Kidney	Testes
8	M	Control	337	9.28	2.47	3.09
8	M	20	241[b]	7.29	1.94	2.98
10	M	10	303[c]	8.60	2.32	3.07
9	M	3	320	8.80	2.40	3.14
9	M	1	323	8.58	2.39	3.18
10	M	0.3	316	8.78	2.32	3.10
10	F	Control	194	5.90	1.58	...
10	F	20	179	5.60	1.55	...
10	F	10	188	5.61	1.53	...
10	F	3	189	5.36	1.49	...
10	F	1	191	6.13	1.47	...
10	F	0.3	194	5.90	1.58	...

[a] Animals fasted overnight. [b] Moderate growth depression. [c] Slight growth depression.

test material in the diet. This conclusion is further substantiated by the fact that there were no adverse effects on the average organ weights and by the lack of any gross or histopathological changes in the tissues, even at the highest dietary intake of 20%.

It may be concluded from these data that Methocel 70HG and 90HG have extremely low repeated oral toxicities.

SUMMARY AND CONCLUSIONS

Groups of 10 male and 10 female rats each were maintained for a period of approximately three months on diets containing 20.0, 10.0, 3.0, 1.0, and 0.3 per cent of Methocel 70HG or Methocel 90HG. A similar set of male and

female rats served as controls and received only the basic ground ration.

There was moderate retardation of growth of the male and female rats receiving the 20 per cent dietary level of Methocel 70HG and a slight growth retardation of the male rats only at the 10 per cent level. In the Methocel 90HG experiment, growth of the female rats was unaffected, even at the 20 per cent dietary level.

Under-nutrition, due to the non-nutritive bulk of the test materials is suggested as being responsible for the effects on growth. No adverse effects as judged by gross appearance, behavior, mortality, blood hematocrit values, average organ weights, or gross and microscopic examination of the tissues were noted in the male and female rats receiving these dietary levels.

The groups of male and female rats that received diets containing 3.0, 1.0, and 0.3 per cent of Methocel 70HG or 90HG showed no evidence, whatsoever, of adverse effects.

It may be concluded that Methocel 70HG and 90HG, like regular Methocel, Methocel 60HG, and Methocel 65HG, are extremely low in chronic oral toxicity. Thus, it is believed that Methocel 70HG and 90HG also are safe for many uses in the food field.

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Stability of Aspirin in Aspirin, Phenacetin, and Caffeine Tablets

By M. R. NAZARETH† and C. LEE HUYCK‡

The stability of aspirin, phenacetin, and caffeine tablets containing starch and talc; starch, alginic acid, and talc; and polyvinylpyrrolidone (PVP), alginic acid, and talc were compared with a control containing starch and magnesium stearate prepared by the precompression method over the period of five weeks at 45°, refrigerator and room temperatures. Taking appearance, disintegration time, and stability of aspirin into consideration, the tablet containing starch and talc prepared by granulating phenacetin, caffeine, and half the starch with 10% starch paste and dried (at 50° for twelve hours to a moisture content of 0.6 per cent) before the addition of aspirin, talc, and the remaining starch proved to be the most satisfactory of those tested.

ASPIRIN combined with phenacetin and caffeine in tablet form is widely used as an analgesic today. Many brands of aspirin-phenacetin-caffeine tablets are commercially available but there is little published data on the stability of the aspirin in this formulation.

Acetylsalicylic acid (1) forms a damp to pasty mass when triturated with acetanilide, acetophenetidin, antipyrine, aminopyrine, methenamine, phenol, or phenyl salicylate. Powders containing acetylsalicylic acid with an alkali salt such as sodium bicarbonate become gummy on contact with atmospheric moisture. Hydrolysis occurs on admixture with salts containing water of crystallization. Solutions of the alkaline acetates and citrates, as well as alkalies themselves, dissolve acetylsalicylic acid, but the resulting solutions hydrolyze rapidly to form salts of acetic and salicylic acids. Sugar and glycerol have been shown to hinder this decomposition. Acetylsalicylic acid very slowly liberates hydriodic

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acid from potassium or sodium iodide. Subsequent oxidation by air produces free iodine.

Ribeiro, *et al.* (2), found that certain lubricants noticeably stearates, increased the degree of decomposition of aspirin in aspirin-phenacetin-caffeine tablets. Moderate moisture content of the phenacetin-caffeine granulation did not increase decomposition of aspirin and the pressure of an ordinary tablet machine was without effect.

Yamamoto and Takahashi (3) reported that high temperature, even at low humidity, caused hydrolysis of aspirin. The effect of external pressure on aspirin was negligible but the presence of a salt of a weak acid accelerated decomposition, especially at high humidity.

This work was undertaken to find an aspirin-phenacetin-caffeine formulation in which the aspirin is stable.

EXPERIMENTAL

Four aspirin-phenacetin-caffeine formulations given in Table I were studied.

TABLE I.—FORMULAS IN GM./1,000 A. P. C. TABLETS

Ingredients	A	B	C	D
Aspirin (20 mesh)	227.0	227.0	227.0	227.0
Phenacetin (powder)	162.5	162.5	162.5	162.5
Caffeine (powder)	32.7	32.7	32.7	32.7
Starch	42.8	42.8	9.0	
Alginic acid			17.8	17.8
Magnesium stearate	10.0			
Talc		8.0	8.0	8.0
PVP				10.0
Total	475.0	473.0	457.0	458.0

Tablets of formula A were prepared by the slugging method. Aspirin, phenacetin, caffeine, and half the magnesium stearate were mixed, slugged, and broken into granules with a No. 14 mesh screen. The remaining magnesium stearate and the starch were mixed with the granules and compressed into tablets weighing 0.475 Gm., using a one-half inch punch.

Tablets of formula B were prepared by granulating phenacetin, caffeine, and half the starch with 10% starch paste. The granules were dried at 50° for twelve hours in a hot air oven and the moisture content determined by a Cenco infrared moisture balance was found to be about 0.6%. Aspirin, talc, and the remaining starch were mixed with the granules and compressed into tablets weighing 0.473 Gm., using a one-half inch punch.

In the case of tablets of formula C, phenacetin, caffeine, and half the alginic acid were mixed and granulated with 10% starch paste. The granulation was dried and the moisture content was found to be approximately 0.4%. Aspirin, talc, and the remaining alginic acid were mixed with the dried granules and compressed into tablets weighing 0.457 Gm., using a one-half inch punch.

Tablets of formula D were prepared by first granulating caffeine, phenacetin, polyvinylpyrrolidone, and half the alginic acid with water. The granulation was dried and the moisture content was found to be about 0.5%. The aspirin, talc, and the remaining alginic acid were mixed with the dried granules and compressed into tablets weighing 0.458 Gm., using a one-half inch punch.

Samples of A, B, C, and D were kept under refrigeration (9–12°), room temperature (25–30°), and 45°. The stability of these samples are shown in Figs. 1–4.

Method of Analysis.—The method of analysis used was essentially that of Pankratz and Bandelin (4). A standard curve was prepared by plotting the absorbance of known concentrations of pure salicylic acid at 525 mμ blanked against 20%

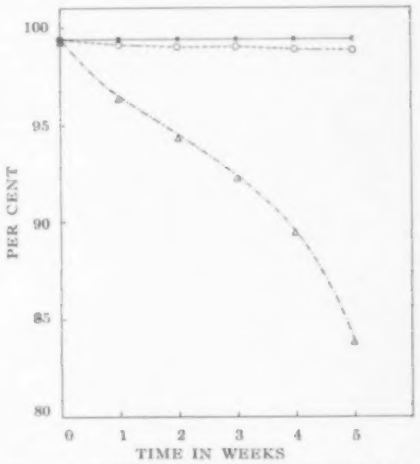

Fig. 1.—Stability of aspirin in formula A at various temperature levels. ×——×, Refrigeration; O- - -O, room temperature; Δ · Δ, 45°.

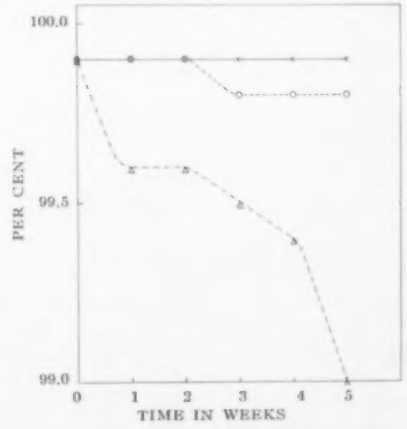

Fig. 2.—Stability of aspirin in formula B at various temperature levels. ×——×, Refrigeration; O- - -O, room temperature; Δ · Δ, 45°.

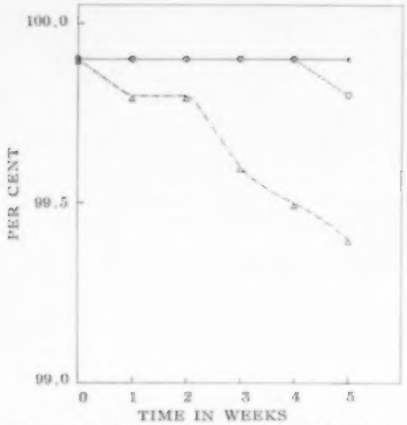

Fig. 3.—Stability of aspirin in formula C at various temperature levels. ×——×, Refrigeration; O—O, room temperature; Δ · Δ, 45°.

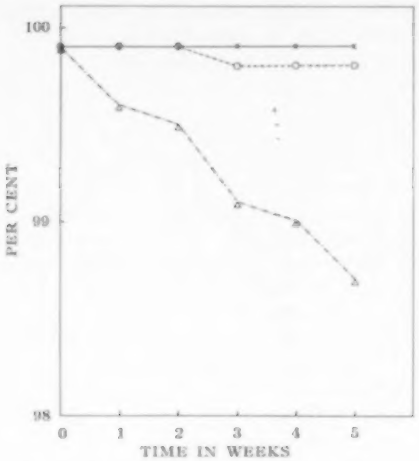

Fig. 4.—Stability of aspirin in formula D at various temperature levels. ×——×, Refrigeration; O- - -O, room temperature; Δ · Δ, 45°.

ethyl alcohol, using a Coleman junior spectrophotometer model 6.

The free salicylic acid in aspirin used in the formulations was found to be 0.05%. The amount of aspirin per tablet in A, B, C, and D was determined as follows: Twenty tablets were accurately weighed and the average weight per tablet was recorded. The tablets were powdered and exactly 1.500 Gm. of the powdered sample was transferred to an Erlenmeyer flask with 25 ml. ethyl alcohol. The flask was well shaken, allowed to stand for ten minutes, and the solution was filtered clear. Twenty-five milliliters of 10% potassium hydroxide solution was added and the mixture was allowed to stand, lightly stoppered on a steam bath, for thirty minutes. It was then cooled and transferred to a 200-ml. volumetric flask. Five milliliters of concentrated hydrochloric acid was added with sufficient distilled water to make up to volume. Ten milliliters of this solution was diluted to exactly 500 ml. A 25-ml. aliquot of this dilution was transferred to a 50-ml. volumetric flask, 10 ml. of ethyl alcohol and 5 ml. of ferric nitrate reagent were added with sufficient distilled water to volume. The absorbance of this solution at 525 mμ was read and compared with the standard. The amount of aspirin/tablet was then determined using the standard curve as a reference. The aspirin content in mg. was found to be A, 227.0; B, 227.0; C, 227.5; and D, 227.2 mg./tablet and was taken to be 227 mg./tablet for all further calculations.

The free salicylic acid present in the freshly prepared tablets was determined as follows: Twenty tablets were accurately weighed and powdered. Ethyl alcohol, 15 ml., was added to 0.500 Gm. of

Table II.—Mg. Decomposed Aspirin/Tablet at Various Storage Conditions for Five Weeks

Time in Weeks	A			B			C			D		
	Ref.[a]	R.T.[b]	45°C.	Ref.	R. T.	45°C.	Ref.	R. T.	45°C.	Ref.	R. T.	45°C.
Initial	1.7	1.7	1.7	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.5
1	1.7	2.1	6.0	0.6	0.6	0.9	0.5	0.6	0.7	0.5	0.6	1.1
2	1.7	2.3	12.9	0.6	0.6	1.1	0.6	0.6	0.9	0.5	0.6	1.5
3	1.7	2.5	17.5	0.6	0.7	1.2	0.6	0.6	1.0	0.6	0.7	2.0
4	1.7	2.7	23.5	0.6	0.7	1.7	0.6	0.6	1.4	0.6	0.7	2.4
5	1.7	2.9	36.2	0.6	0.7	2.3	0.6	0.7	1.8	0.6	0.7	3.1

[a] Ref., refrigerated (9–12°). [b] R. T., room temperature (25–30°).

Table III.—Per Cent of Theoretical Amount of Aspirin/Tablet at Various Storage Conditions for Five Weeks

Time in Weeks	A			B			C			D		
	Ref.[a]	R. T.[b]	45°C.	Ref.	R. T.	45°C.	Ref.	R. T.	45°C.	Ref.	R. T.	45°C.
Initial	99.4	99.4	99.4	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9
1	99.4	99.1	96.5	99.9	99.9	99.6	99.9	99.9	99.8	99.9	99.9	99.6
2	99.4	99.0	94.5	99.9	99.9	99.6	99.9	99.9	99.8	99.9	99.9	99.5
3	99.4	99.0	92.4	99.9	99.8	99.5	99.9	99.9	99.6	99.9	99.8	99.1
4	99.4	98.9	89.6	99.9	99.8	99.4	99.9	99.9	99.5	99.9	99.8	99.0
5	99.4	98.8	84.0	99.9	99.8	99.0	99.9	99.8	99.4	99.9	99.8	98.7

[a] Ref., refrigerated (9–12°). [b] R. T., room temperature (25–30°).

the powdered sample in an Erlenmeyer flask. The flask was shaken well and allowed to stand for ten minutes, after which the solution was immediately filtered clear into a 100-ml. volumetric flask. Five milliliters of ferric nitrate reagent was added and distilled water was added to volume. The absorbance of the solution was then read at 525 mμ when blanked against 20% ethyl alcohol. The amount of free salicylic acid present was read from the standard curve and the per cent labeled amount recorded.

In the case of formulation A the solution became turbid on making it up to volume with distilled water. The turbidity was probably due to the dissolved magnesium stearate being thrown out of solution by water. The solution was filtered before reading the absorbance.

The hardness of the tablets as determined by the Strong-Cobb tablet hardness tester was found to be A, 7 Kg.; B, 10 Kg.; C, 9 Kg.; and D, 10 Kg. The disintegration time of the freshly prepared tablets was found to be A, fifteen seconds; B, twenty seconds; C, ten minutes; and D, twelve minutes.

The per cent labeled amount of A, B, C, and D at the three temperature levels was determined weekly throughout the period of study and these results are recorded in Tables II and III.

DISCUSSION

Although it is stated that aspirin forms a pasty mass when in contact with acetophenetidin (1), the diluents in formula B are obviously sufficient to hinder this reaction. It has also been reported that the deterioration of aspirin (1) is hindered by sugar and glycerol, and this may help to explain the stability of this formulation.

Formulation A had a lower initial value than formulations B, C, and D. The lower value is probably due to the presence of magnesium stearate in the formulation. This is in agreement with the report of Ribeiro, *et al.* (2). Magnesium stearate was used as a lubricant because talc, liquid paraffin, or a combination of the two did not slug well.

Formulation A did not deteriorate when kept under refrigeration. At room temperature it deteriorated slowly, and the final value after a five week aging period was 98.8% of the labeled amount. At 45° decomposition of aspirin was more rapid. There was a distinct odor of acetic acid from the second week onward, and the final value was 84.0% of the labeled strength.

Formulation B showed no deterioration under refrigeration and room temperature. At 45° there was slight decomposition in that the final value was 99.0% of the labeled amount.

Formulation C also showed no apparent deterioration under refrigeration and room temperature. At 45° there was a very slight deterioration and the tablets took on a faint buff color.

Formulation D showed hardly any deterioration under refrigeration and room temperature. At 45° it also decomposed very slowly to a final value of 98.7% of the labeled amount and the tablets showed a slight discoloration.

Taking appearance, disintegration time, and stability of aspirin into consideration, formula B emerges as the best of the four formulations studied and can easily be used in the preparation of tablets of aspirin, phenacetin, and caffeine.

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Incorporation of 2-C^{14}-Sodium Acetate into Sterol and Pentacyclic Triterpenes of *Salvia officinalis*

By HAROLD J. NICHOLAS

C^{14} from 2-C^{14}-sodium acetate presented to *S. officinalis* L. by absorption into isolated leaves and into flowering stems devoid of leaves was incorporated into both sterol and acidic triterpenes of each plant section.

IN STUDYING the incorporation of precursors into sterols and triterpenes of higher plants it would be of interest to know whether all organs of the plant possess this synthetic capacity. At our present state of knowledge such information is not available in the literature, especially in regard to biosynthesis of the pentacyclic triterpenes (1). The present study was conducted primarily to ascertain if isolated organs of *S. officinalis* L. would incorporate 2-C^{14}-sodium acetate into sterol (β-sitosterol) and pentacyclic triterpenes (oleanolic acid and ursolic acid I). These substances have previously been reported present in *S. officinalis* L. (2, 3). Presenting labeled precursors to higher plants quantitatively frequently poses a problem. For our preliminary work we

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TABLE I.—INCORPORATION OF 2-C^{14}-SODIUM ACETATE INTO CRUDE STEROL AND TRITERPENE FRACTIONS OF *S. officinalis* ORGANS

Plant Organ	Crude Sterol Fraction		Crude Triterpene Fraction			
	C^{14} Content		Ursolic Acid I		Oleanolic Acid	
	Wt., mg.	c. p. m.	Wt., mg.	C^{14}, c. p. m.	Wt. mg.	C^{14}, c. p. m.
Flowers and stem	2.2	26,950	2.5	953,600	2.5	99,200
Leaves only	2.0	13,750	0.8	965,800	2.2	203,960

have chosen the method of Krotkov and Barker (4), who studied the distribution of radioactivity into various fractions following the absorption of solutions of C^{14}-labeled acetate into cut sections of tobacco leaves while stimulated by light. This method has the advantage of permitting quantitative absorption of the solutions to be absorbed, and in a relatively short period of time.

EXPERIMENTAL

Materials and Methods.—All solvents were A. R. grade or equivalent. Sterol and triterpene samples for dilution were from sources previously described (5). The 2-C^{14}-sodium acetate was purchased from Isotopes Specialties, Inc., Burbank, Calif., and had a specific activity of 6.02 mc./*m*M. All C^{14} determinations were performed with a windowless, gas-flow counter on samples plated as infinitely thin layers on nickel planchets 2.5 cm. in diameter. All samples were counted for a sufficient length of time to insure a counting error of 5% or less. Plants were grown from seeds obtained from the Vaughn Seed Company, Chicago, Ill.

Incorporation of 2-C^{14}-Sodium Acetate into Sterol and Triterpene of Flowers and Main Stem.—A flowering section of *S. officinalis* L. was cut with a razor well below the flowering area and all leaves were severed at the base of the petioles. The cut end of the stem was immersed in a small glass vessel containing 1.0 ml. of 2-C^{14}-sodium acetate in water (a total of 1.1×10^8 c. p. m.). During the absorption period, about twenty minutes, the section was illuminated according to Krotkov and Barker (4). Immediately after the acetate solution was absorbed, 1 ml. of distilled water was placed in the container, after which the section was allowed to stand overnight without illumination. The portion immersed in the solution was then rinsed in tap water and the section was allowed to dry at room temperature for two weeks. It was then cut into small pieces and extracted exhaustively with ethanol. The concentrate of the ethanol extracts was fractionated into nonsaponifiable and potassium hydroxide-soluble, as previously outlined (2). Only that portion of the alcohol concentrate soluble in ethyl ether was examined. The plant material after extraction weighed 0.290 Gm. From the nonsaponifiable fraction sterol was precipitated as the digitonide according to Sperry and Webb (6) and the digitonide subsequently converted to free sterol by the pyridine method (7). The C^{14}-content of the crude sterol mixture is shown in Table I. The crude acidic triterpene fraction was chromatographed on Whatman No. 1 paper as previously described (2). The forty-eight-hour overflow was collected as the oleanolic acid fraction and the material at the starting line eluted for the ursolic acid fraction. Yield and C^{14}-content of the crude fractions are shown in Table I also. To the crude sterol fraction, 100.0 mg. of β-sitosterol was added. To the crude overflow material, 100.0 mg. of oleanolic acid, and to the material eluted from the starting line, 100.0 mg. of ursolic acid I was added. Each mixture was then crystallized twice, the β-sitosterol from acetone-methanol, the acids from aqueous or absolute ethanol. Approximately 50 mg. of each sample was at this point taken for acetylation; the remainder was reserved for further crystallization. Acetylation was performed by refluxing each sample one-half hour with 3.0 ml. acetic anhydride and anhydrous pyridine, respectively. The cooled mixture was diluted with water, extracted with ether, and then washed successively with water, 10% hydrochloric acid, water, 5% sodium bicarbonate, and water. The ether was distilled and the product recrystallized, the β-sitosterol acetate from acetone-methanol, the acetylated acids from aqueous ethanol. The free compounds were recrystallized eight times, the acetates four times. There was no change in specific activity of the free compounds or acetates after two crystallizations (Table II). Saponification of the acetates and two crystallizations of the free compound so-obtained gave material with a specific activity in agreement with that obtained by direct crystallization without acetylation.

TABLE II.—INCORPORATION OF 2-C^{14}-SODIUM ACETATE INTO β-SITOSTEROL, URSOLIC ACID I, AND OLEANOLIC ACID OF *S. officinalis* ORGANS

Plant Organ	Specific Activity, c. p. m. per mg.					
	β-Sitosterol		Ursolic Acid		Oleanolic Acid	
	Free	Acetate	Free	Acetate	Free	Acetate
Flowers and stem	184	167	1,177	1,126	53	40
Leaves only	96	84	141	129	192	180

Incorporation of 2-C^{14}-Sodium Acetate into Sterol and Triterpene of Leaves.—Four large leaves from the same plant used for the previous experiment were immersed at the severed end in 1.0 ml. of the 2-C^{14}-sodium acetate solution and otherwise treated as indicated for the flowering stem section. The plant material after ethanol extraction weighed 0.197 Gm. Yield and C^{14}-content of crude sterol and triterpene fractions and respective diluted and recrystallized fractions are shown in Tables I and II.

RESULTS AND DISCUSSION

C^{14} was incorporated from 2-C^{14}-sodium acetate into both sterol and acidic triterpenes of flowers and stem devoid of leaves or into isolated leaves of *S. officinalis* L. (Tables I and II). Because it was necessary to resort to considerable dilution of the various fractions with nonradioactive material in

order to obtain sufficient product for repeated crystallizations, some caution must be observed in interpreting the specific activity figures shown in Table II. It is obvious, however, that only a relatively small proportion of the radioactivity in the crude acidic triterpene fractions was contributed by the triterpenes themselves. The generally higher specific activity of β-sitosterol and ursolic acid I in the flower-stem section is probably a contribution largely from the flower parts themselves, this being a general phenomenon observed in our studies thus far. On this account and perhaps others, it seems best to delay any further interpretation of the interesting high specific activity shown by ursolic acid I in the flower-stem section. The chromatographic procedure for separating oleanolic acid and the ursolic acids does not separate ursolic acids I and II, the latter both remaining at the starting line. Since both of the ursolic acids are present in *S. officinalis* L. (3) this represents somewhat of a problem for tracer work. For the work herein described it seems highly probable that ursolic acid II, possessing a much greater solubility than ursolic acid I, was removed in the crystallizations described and that the specific activity indicated for ursolic acid I is indeed due to this compound.

In a number of as yet unpublished experiments it has been found that 2-C^{14}-acetate or -mevalonate presented to several Labiates in the manner described gave rise to C^{14}-labeled sterol and triterpenes in all parts of the plants examined. Barring actual transport of sterol or triterpene during the experiments (which seems highly unlikely at this time), this information together with the present report gives strong evidence in favor of sterol and triterpene synthesis *in situ* for either leaf, stems, or flowers. It should be added that because of the diverse pathway taken by acetate in its metabolism, the present data are not informative regarding the mode of biosynthesis of either sterol or triterpene.

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Notes

Saccharin Derivatives V. Synthesis of 5-Aminosaccharin and Related Compounds

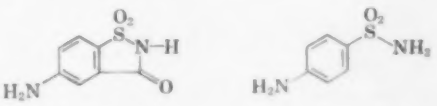
By ALTON WARREN and GLENN H. HAMOR

The synthesis of the following compounds is reported: 5-nitrosaccharin, 5-aminosaccharin, 5-acetamidosaccharin, 2-methyl-5-nitrosaccharin, and 2-ethyl-5-nitrosaccharin. These substances lacked a sweet taste, being either bitter or tasteless.

AS PART of a continuing study of saccharin derivatives, the preparation of 5-aminosaccharin and related compounds was desired. Various saccharins have been reported to have local anesthetic (1) or analgesic and antihistaminic (2) activity. The compounds were also synthesized to be included in a forthcoming paper on the relationship of chemical structure to taste in the saccharin series.

The 5-nitrosaccharin was needed to further substantiate previous work in which attempts to prepare 5-nitrosaccharin, using procedures described in the literature, gave instead a mixture of two nitrosaccharins which was shown to consist of 6-nitrosaccharin and 4-nitrosaccharin (3).

Our final reason for synthesis of 5-aminosaccharin will have primarily historical meaning. Backeberg and Marais in the early 1940's were interested in the preparation of 5-aminosaccharin because of its structural similarity to sulfanilamide, as shown below (4). They obtained 5-acetamidosaccharin but could not get the desired compound on deacetylation attempts. With the knowledge that sulfanilamide exerts its bacteriostatic action by competing with *p*-aminobenzoic acid (5), there should be no logical reason for expecting 5-aminosaccharin to have similar activity. However, 5-aminosaccharin was synthesized to fill this void in the history of sulfanilamide-like compounds.



The parent compound, 5-nitrosaccharin, from which all the others were prepared, was synthesized according to the following series of reactions:

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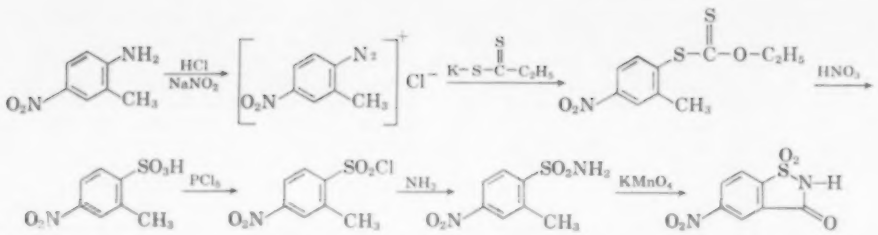
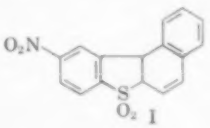


Reduction of 5-nitrosaccharin by hydrogen with palladium-on-carbon catalyst, followed by acetylation, gave 5-aminosaccharin and 5-acetamidosaccharin. N-Alkylation of 5-nitrosaccharin gave 2-methyl-5-nitrosaccharin and 2-ethyl-5-nitrosaccharin. As the above reactions are well-known, only general procedures and physical properties of the saccharin derivatives will be listed in the experimental section.

After completion of our synthesis of 5-nitrosaccharin, the report of Davies and Porter of the University of Melbourne describing the synthesis of 5-nitrosaccharin appeared in the literature (6). The compound was prepared for use as supporting evidence for the structure of 10,11-dihydro-6-nitro-9-thia-3,4-benzofluorene-9,9-dioxide (I). Oxidation



of this substance with potassium permanganate gave 5-nitro-2-sulfobenzoic acid which was converted to 5-nitrosaccharin and compared with the 5-nitrosaccharin prepared from an authentic sample of 5-nitro-2-sulfobenzoic acid.

This paper then reports the preparation of three new compounds: 5-aminosaccharin, 2-methyl-5-nitrosaccharin, and 2-ethyl-5-nitrosaccharin, along with the known 5-nitrosaccharin and 5-acetamidosaccharin. These substances lacked a sweet taste, being either bitter or tasteless.

EXPERIMENTAL[1]

5-Nitrosaccharin.[2]—This compound was prepared by alkaline potassium permanganate oxidation of 4-nitro-*o*-toluene sulfonamide by the method used by Noyes (7) to synthesize 6-nitrosaccharin. The 4-nitro-*o*-toluenesulfonamide, m. p. 157° (reported m. p. 157°) (8), was synthesized according to the series of reactions shown above. Recrystallization of the 5-nitrosaccharin from ethyl acetate gave yields of approximately 30% of fine, yellowish-white crystals, m. p. 212–214° (reported m. p. 228°)[3] (6).

Anal.[4]—Calcd. for $C_7H_4N_2O_5S$: C, 36.84; H, 1.77. Found: C, 36.83; H, 1.85.

5-Aminosaccharin.—Reduction of 5-nitrosaccharin using hydrogen with palladium-on-charcoal catalyst, according to standard procedures (3), followed by recrystallization of the product from water gave crystalline 5-aminosaccharin (77%) m. p. 291–293°. Dilute solutions of the compound in ethanol displayed a bluish fluorescence.

Anal.—Calcd. for $C_7H_6N_2O_3S$: C, 42.42; H, 3.05. Found: C, 42.36; H, 3.10.

5-Acetamidosaccharin.—5-Aminosaccharin was acetylated using acetic anhydride, with the aid of pyridine, using published procedures (3, 9). Almost quantitative yields of cream-colored crystalline product, m. p. 304–305° (capillary tube) (reported m. p. 299°) (4), was obtained. This 5-acetamidosaccharin was identical to that of Backeberg and Marais as shown by no depression of a mixed melt.[5]

Anal.—Calcd. for $C_9H_8N_2O_4S$: C, 44.99; H, 3.37. Found: C, 44.89; H, 3.47.

2-Methyl-5-nitrosaccharin.—The method of Merritt, Levey, and Cutter (10) was employed for the preparation of this compound (1). Yellow to white needle crystals of 2-methyl-5-nitrosaccharin, m. p. 169–170° (23%) were obtained.

Anal.—Calcd. for $C_8H_6N_2O_5S$: C, 39.67; H, 2.50. Found: C, 40.04; H, 2.51.

2-Ethyl-5-nitrosaccharin.—This compound was prepared by the same procedure used for the 2-methyl derivative. Yellowish-white, crystalline 2-ethyl-5-nitrosaccharin, m. p. 114–115° (28%) was obtained.

Anal.—Calcd. for $C_9H_8N_2O_5S$: C, 42.18; H, 3.15. Found: C, 42.41; H, 3.06.

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[1] Melting points were taken with a Fisher-Johns melting point apparatus and are uncorrected.

[2] *Chem. Abstr.* nomenclature: 5-nitro-1,2-benzisothiazolin-3-one-1,1-dioxide.

[3] When a sample of the material melting at 228° becomes available, a mixed melt with our sample will be run.

[4] Analyses were performed by Elek Micro Analytical Laboratories, Los Angeles, Calif.

[5] The authors are indebted to J. L. C. Marais, University of Witwatersrand, Johannesburg, South Africa, for supplying the sample of 5-acetamidosaccharin.

Occurrence of *d*-Sparteine in *Baptisia leucantha* T. and G.

By S. MORRIS KUPCHAN and NORMAN A. DAHLE†

BAPTISIA LEUCANTHA T. and G., of widespread occurrence in eastern North America, has been used among the Indians as a cathartic, strong emetic, and in small doses as a laxative (1). The genus *Baptisia* includes several species reported to have caused poisoning in animals (2, 3). The sole recorded observation concerning the alkaloids of *B. leucantha* appears to have been the reported detection of the presence of cytisine by means of a microchemical test (4).

The work reported herein concerns a preliminary investigation of the alkaloids extracted from the aerial portion of *B. leucantha* collected in July 1959 in central Wisconsin. The dried and ground plant was extracted with methanol and processed for alkaloid content in the usual manner (5). Paper chromatographic examination of the crude alkaloidal mixture revealed the presence of two alkaloids in approximately 80:20 proportion. Distillation of the crude alkaloid mixture under reduced pressure yielded an 0.29% yield of *d*-sparteine, the major alkaloid. The latter compound was characterized as the cyrstalline dipicrate derivative. Attempts at isolation of the minor alkaloid by fractional crystallization of the picrate prepared from the alkaloid mixture prior to distillation were unsuccessful.

d-Sparteine (pachycarpine) is pharmacologically active; it affects the secretory and motor activities of the stomach (6) as well as the contraction of the uterus and the protective-inhibitory processes of the cerebral cortex (7). It appears reasonable that the *d*-sparteine content of *B. leucantha* may have contributed to the aforementioned properties attributed to the plant.

EXPERIMENTAL

Melting points were determined on a Fisher-Johns hot stage. Infrared spectra were determined on a Baird model 4-55 double beam infrared recording spectrophotometer in Nujol mulls.

Isolation and Identification of *d*-Sparteine. The dried and ground aerial portion of *B. leucantha* (1,500 Gm., collected in July 1959 in fruit in Central Wisconsin)[1] was extracted exhaustively with methanol in a Soxhlet extractor. The methanol was largely evaporated under reduced pressure, and the concentrate was diluted with water, made acid to Congo red with hydrochloric acid, and kept on the steam bath for nine hours. The mixture was cooled, filtered, and the insoluble cake warmed again with dilute acid, cooled, and filtered. The combined aqueous acid filtrate was extracted with ether and the ether extract was rejected. The aqueous acid solution was made alkaline with potassium hydroxide solution and extracted continuously with chloroform in a Rinco extractor. The chloroform extract was evaporated to dryness, and the residual base dissolved in warm dilute hydrochloric acid, and the cooled solution was filtered through charcoal. The aqueous solution was made alkaline with ammonia and repeatedly extracted with chloroform. The chloroform extract was evaporated to dryness under reduced pressure.

Paper chromatographic analysis of the crude alkaloid mixture indicated the presence of two alkaloids in approximately 80:20 proportion.[2] Distillation of the crude alkaloid mixture under reduced pressure yielded, as major fraction, 4.4 Gm. (0.29%) of colorless oil, b. p. 80–90° (0.3 mm.); $[\alpha]_D^{25}$ + 15° (c 2.00, abs. alc.). Paper chromatographic analysis revealed the homogeneity of the material.

The distilled base (840 mg.) was dissolved in methanol and added to a methanolic solution of picric acid. On cooling, a picrate separated. After two recrystallizations from methanol, the picrate was obtained as prismatic needles, m. p. 206–208°. The infrared spectrum was identical with that of an authentic sample of *l*-sparteine dipicrate.[3] The latter fact and the aforementioned positive optical rotation of the free base led to identification of the *B. leucantha* base as *d*-sparteine. In accordance with expectation (5), the melting point of the *d*-sparteine dipicrate was depressed by admixture of *l*-sparteine dipicrate.

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[1] The authors thank Professor H. H. Iltis of the University of Wisconsin Herbarium for confirming the identity of the plant.
[2] The method involved the use of paper pretreated with phosphate-citrate buffer at pH 3.5 and the detection of alkaloidal spots with a chloroform solution of bromophenol blue. The solvent system was the layer of a mixture of *n*-butanol:acetic acid:water (63:27:10 by volume) prepared by shaking well and allowing to stand at room temperature for two days.
[3] The authors thank Professor E. E. van Tamelen of the Department of Chemistry for an authentic sample of *l*-sparteine dipicrate.

Steroids VIII. 3,6-Diaza-A,B-bishomo-5α-cholestane-4,7-dione

Sir:

Recently Shoppee and Sly (1) conducted the Beckmann rearrangement of 5α-cholestan-3-one oxime and 5β-cholestan-3-one oxime and proved the structures of the products obtained to be 3-aza-A-homo-5α-cholestan-4-one and 3-aza-A-homo-5β-cholestan-4-one, respectively. We prepared compounds in the aza-B-homo-5α-cholestane series, by Schmidt reaction with 3β-acetoxy-5α-cholestan-6-one and 3β-acetoxy-5α-cholestan-7-one. The structures of the corresponding products have not been established as yet, but on the basis of theoretical considerations, they are expected to be 3β-acetoxy-6-aza-B-homo-5α-cholestan-7-one and 3β-acetoxy-7a-aza-B-homo-5α-cholestan-7-one (2).

Now we have obtained a compound (II) in the A,B-bishomo-5α-cholestane series, having one nitrogen in each of the enlarged ring systems. To our knowledge, this is the first steroid of this type to be prepared.

5α-Cholestane-3,6-dione (3) (I) was submitted to the Schmidt reaction using polyphosphoric acid as the solvent and catalyst (4). A product m.p. >300° [1] (decompn.) was obtained.

Anal.—Calcd. for $C_{27}H_{46}N_2O_2$ C, 75.30; H, 10.77; N, 6.51. Found: C, 75.66; H, 10.69; N, 6.42 $[\alpha]_D^{25} = +8.0°$ (C, 0.5% in chloroform). Infrared spectrum: 2.92 μ (free NH); 3.08 μ (associated NH); 6.01 μ (lactam C=O). It is tentatively considered to be 3,6-diaza-A,B-bishomo-5α-cholestane-4,7 dione (II).

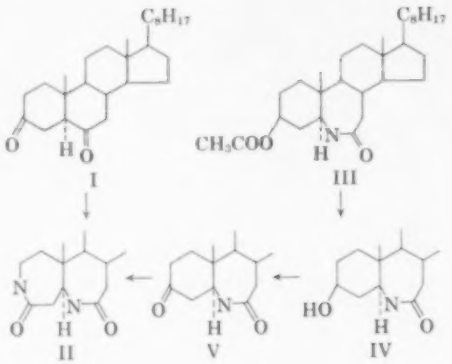
3β-Acetoxy-6-aza-B-homo-5α-cholestan-7-one (III), m. p. 222.4–225.0° (corr.) was prepared (2).

Anal.—Calcd. for $C_{29}H_{49}NO_3$: C, 75.77; H, 10.74; N, 3.05. Found: C, 75.43; H, 10.58; N, 3.14. $[\alpha]_D^{25} = +31.5°$ (C, 1.0% in chloroform). Infrared spectrum: 2.92 μ (free NH); 5.78 μ (ester C=O); 6.01 μ (lactam C=O); 8.01 μ (acetate C—O—).

On basic hydrolysis the compound gave 3β-hydroxy-6-aza-B-homo-5α-cholestan-7-one (IV), m. p. 278–281°.

Anal.—Calcd. for $C_{27}H_{47}NO_2$: N, 3.35. Found: N, 3.39. Infrared spectrum: 2.75 μ (free OH); 2.92 μ (free NH); 6.01 μ (lactam C=O).

[1] Melting points are uncorrected and rotations were determined in chloroform.



Compound IV was oxidized with chromic acid in acetic acid to yield 6-aza-B-homo-5α-cholestane-3,7-dione (V), m. p. 238–243°.

Anal.—Calcd. for $C_{27}H_{45}NO_2$: N, 3.37. Found: N, 3.67. Infrared spectrum: 2.92 μ (free NH); 5.79 μ (keto C=O); 5.98 μ (lactam C=O).

Compound V on Schmidt reaction gave a product (II) m.p. >300°; $[\alpha]_D^{25} = +10.0°$ (C, 1.0% in chloroform). Infrared spectrum: 2.92 μ (free NH); 3.08 μ (associated NH); 6.01 μ (lactam C=O). This was shown to be identical with the product obtained from 5α-cholestane-3,6-dione by Schmidt reaction. The identity was established on the basis of mixed melting point, which showed no depression, and comparison of infrared spectra, which were similar.

The preparation of II from 6β-acetoxy-5α-cholestan-3-one, by stepwise reactions, is in progress in this laboratory.

The details will be published in a full paper on this problem.

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For paper VII in this series see Doorenbos, N. J., and Huang, C. L., *J. Org. Chem.*, in press.

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